

THE ROLE OF NEUTROPHILS
IN THE IMMUNOPATHOGENESIS
OF DENTAL DISEASES

W.K. SEOW

THE ROLE OF NEUTROPHILS IN THE IMMUNOPATHOGENESIS
OF DENTAL DISEASES

by

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STATEMENT OF AUTHENTICITY

The work presented in the thesis is, to the best of the candidate's knowledge and belief, original, except as acknowledged in the text, and the material has not been submitted, either in whole or in part, for a degree at this or any other university.

A handwritten signature in black ink, appearing to read 'W. Kim Seow', with a long horizontal flourish extending to the right.

W. Kim Seow

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PUBLICATIONS FROM THE THESIS

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2. Seow WK, Thong YH (1987). Modulatory effects of Streptococcus mutans on human neutrophil adherence and deoxyglucose uptake. Int Arch Allergy appl Immunol 82:40-45.
3. Seow WK, Thong YH (1986). Modulation of polymorphonuclear leukocyte adherence by pulpotomy medicaments: effects of formocresol, glutaraldehyde, eugenol and calcium hydroxide. Pediatr Dent 8:16-21.
4. Seow WK, Seymour GJ, Thong YH (1987). Direct modulation of human neutrophil adherence by coaggregating periodontopathic bacteria. Int Arch Allergy appl Immunol 83:121-128.
5. Seow WK, Thong YH (1987). Bacteria-phagocyte interactions: Fusobacterium-induced secretion of a neutrophil self-regulatory factor. Immunol Lett 14:95-101.

6. Seow WK, Thong YH (1986). Augmentation of polymorphonuclear leukocyte adherence by interferon. *Int Arch Allergy appl Immunol* 79:305-311.
7. Seow WK, Thong YH, McCormack JG, Ferrante A (1987). Lymphocyte-neutrophil interactions: opposite effects of interleukin-2 and tumour necrosis factor-beta (lymphotoxin) on human neutrophil adherence. *Int Arch Allergy appl Immunol* (in press).
8. Seow WK, Thong YH, Ferrante A (1987). Macrophage-neutrophil interactions: contrasting effects of the monokines interleukin-1 and tumour necrosis factor (cachetin) on human neutrophil adherence. *Immunology* 62:357-362.
9. Seow WK, Li SY, Thong YH (1986). Inhibitory effects of tetrandrine on human neutrophil and monocyte adherence. *Immunol Lett* 13:83-88.
10. Seow WK, Ferrante A, Li SY, Thong YH (1987). Anti-phagocytic and anti-oxidant properties of the plant alkaloid tetrandrine. *Int Arch Allergy appl Immunol* (in press).

ABSTRACT

The work in this thesis was organised as a series of investigations culminating in the publication of several papers which are summarised below.

The important role of the neutrophil in defence of the oral cavity was shown in investigations of the direct interaction of neutrophils and four serotypes (a,c,dg,e) of *Streptococcus mutans*. The results show that only serotype c has the capacity to suppress neutrophils compared to the other serotypes. This property is mediated through receptors in the bacterial cell wall as its disruption by heat, ultrasonication and formalin resulted in abrogation of the effect. As serotype c is the most commonly isolated serotype of *Streptococcus mutans* world-wide, the ability of the organism to circumvent the neutrophils may contribute to its increased propensity to colonise the oral cavity.

A possible role for the neutrophil in mediating inflammation and tissue injury in the dental pulp was investigated by examining the effects of common pulpotomy medicaments on neutrophils. The results showed that formocresol, eugenol, and calcium hydroxide caused lysis of neutrophils at high concentrations but activa-

tion of neutrophil adherence at low concentrations. In contrast, glutaraldehyde did not produce neutrophil lysis at high concentrations nor did it cause neutrophil activation at low concentrations. These findings correspond to previous histological studies which found that formocresol, eugenol and calcium hydroxide, but not glutaraldehyde, can cause inflammatory destruction of pulpal tissues.

With regard to periodontal disease, it was shown that direct interaction of neutrophils and *Fusobacterium nucleatum*, a common periodontal pathogen, produced consistent enhancement of neutrophil adherence. In contrast, suppression of neutrophil adherence was observed after direct interactions between neutrophils and *Bacteroides gingivalis* or *Actinomyces viscosus*. The effects were abrogated by alteration of bacterial structural integrity with heat, formalin, ultrasonic disruption as well as by incubation of bacterial cells with D-galactose, indicating that glycoprotein receptors are probably involved. In addition, coaggregation experiments showed that permutations involving *Fusobacterium nucleatum* always resulted in enhancement of neutrophil adherence.

In further investigations of neutrophil-bacterial interactions, it was shown that direct interaction with

Fusobacterium nucleatum also resulted in the secretion of a neutrophil self-regulatory factor(s). The secretion of this factor was bacteria-specific as direct interaction with *Bacteroides gingivalis* resulted in secretion of a soluble factor with inhibitory effects on neutrophil adherence. The secretion of the neutrophil enhancing factor induced by *Fusobacterium* depended on the integrity of the bacterial cell wall and occurred within 15 min. of bacteria-neutrophil interaction. The factor was sensitive to trypsin and heat treatment and ultrafiltration experiments showed that it has a molecular weight of between 10,000 and 30,000 daltons. Its biological role may be that of a molecular mediator for the recruitment of resting neutrophils so as to amplify the immunological and inflammatory response.

These direct neutrophil-bacterial interactions have significance not only in the determination of the ability of certain periodontopathic organisms to colonise the periodontium but also in the initiation and sustenance of chronic inflammatory periodontal diseases.

To further elucidate the role of the neutrophils, their biological control by several lymphokines and monokines were investigated.

Augmentation of human neutrophil adherence by alpha and gamma interferon occurred as early as 2 mins. after incubation. There was synergism between alpha and gamma interferon, but not between two subtypes of alpha interferon on augmentation of adherence, indicating that alpha and gamma interferon act on different receptors on the neutrophils. Heat treatment at 65°C for 30 min. abolished the effect of interferon on adherence.

Human neutrophil adherence was also enhanced by recombinant human tumour necrosis factor-beta (TNF- β) but suppressed by recombinant human interleukin-2 (IL-2). The opposite effects of these two lymphokines were observed over a range of concentrations consistent with their other biological activities, occurred within 15 min. of incubation, and were still evident after 60 min. Pre-treatment of neutrophils with both IL-2 and TNF- β resulted in adherence values intermediate between the enhanced and depressed values obtained with the individual lymphokines. IL-2 suppressed the stimulatory effects of both the chemotactic peptide FMLP and the phorbol ester PMA.

Incubation of human neutrophils with recombinant human interleukin-1 (IL-1) resulted in suppression of neutrophil adherence. In contrast, similar treatment with recombinant human tumour necrosis factor- α (TNF- α),

cachectin) resulted in enhancement of neutrophil adherence. Simultaneous addition of these two monokines resulted in intermediate values between suppression by IL-1 and enhancement by $\text{TNF}\alpha$. The stimulatory effects of the chemotactic peptide FMLP and the phorbol ester PMA were ameliorated by IL-1 but augmented by $\text{TNF}\alpha$.

In other studies, the pharmacological control of neutrophils by tetrandrine, a benzylisoquinoline alkaloid useful in the treatment of silicosis was investigated. A broad range of human neutrophil activities was examined in vitro. Adherence, random movement, chemotaxis and phagocytosis were significantly suppressed. There was minimal but significant inhibition of lysosomal enzyme secretion from specific (secondary) but not azurophil (primary) granules. The same concentration of tetrandrine (10 $\mu\text{g/ml}$) caused marked depression of hexose-monophosphate shunt activity and hydrogen peroxide production, but inhibition of superoxide anion generation was observed even at a concentration of 0.1 $\mu\text{g/ml}$. This discrepancy was attributed to the capacity of tetrandrine to scavenge oxygen radicals, as shown by experiments using hypoxanthine-xanthine oxidase to generate superoxide. These potent anti-phagocytic and anti-oxidant properties of tetrandrine may account for some of its remarkable anti-inflammatory effects, which may have useful clinical

applications.

In conclusion, this thesis has further elucidated the role of the neutrophils in oral diseases in the following areas. In dental caries, it has provided a possible explanation of the increased propensity of serotype c *Streptococcus mutans* to colonise the oral cavity. In pulp diseases, it has shown that commonly used pulpotomy medicaments may be the initiating factors responsible for the unsatisfactory chronic inflammatory states usually seen after pulpotomies. In periodontal diseases, it has provided further elucidation of the roles of the neutrophils in the colonisation of periodontopathic bacteria as well as the initiation and sustenance of chronic inflammatory diseases. In addition, the work in this thesis has further extended the knowledge of the effects of several lymphokines and monokines on neutrophils. As well, the investigations of the properties of the novel anti-inflammatory drug, tetrandrine may pave the way for the development of a new potent and efficacious anti-inflammatory agent that is suitable for many common chronic inflammatory conditions which are not well controlled by currently available anti-inflammatory agents.

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CHAPTER ONE

REVIEW OF THE LITERATURE

Introduction

One of the most important discoveries in host-defence mechanisms is that of Ilya Metchnikoff in 1882, who first observed that a foreign body introduced into a starfish caused the accumulation of motile cells around it (Klebanoff & Clark, 1978). This observation led to the conceptual breakthrough in the role of motile, engulfing cells in host defence. These cells, named "phagocytes" by Metchnikoff for their engulfing actions, are now known to form the first line of defence against extracellular microorganisms. It is now known that there are two main phagocytic cells in the body, the polymorphonuclear neutrophils which are circulating cells that accumulate early in sites of inflammation, and the mononuclear phagocytes found in fixed tissues as well as in the circulation, which accumulate

later at inflammatory foci. In the decades since their discovery, vast increases in knowledge about these cells have extended their roles from a primary one of defence to include several others such as inflammation and tissue injury as well as immunoregulation.

However, despite significant advances in research of the phagocytic cells in general, their roles in dental diseases are not well appreciated. The oral cavity is an environment where there is constant interaction of microbes and phagocytic cells, in particular, the neutrophils, yet the roles of these cells apart from the primary function of defence, are not well defined. The aim of this project is to elucidate the roles of the neutrophil in a number of dental diseases, namely dental caries, pulp inflammation and periodontal disease.

The first chapter of the thesis reviews the relevant literature and the second describes a new method developed to assay neutrophil function. In the third chapter, the role of the neutrophil in defence is illustrated in a study showing that depression of neutrophil responses by a particular strain of *Streptococcus mutans* allows its preferred colonisation in the dental plaque. The fourth chapter discusses the importance of the neutrophil in inflammation and tissue

injury as demonstrated in our study on the effects of pulp medications on these cells. In the next two chapters, the role of neutrophils in the pathogenesis of periodontitis is investigated using the model of direct interaction between neutrophils and periodontopathic bacteria. Chronic periodontitis is a complex disease with immunological and inflammatory components in its pathogenesis. This intimate relationship between inflammatory and immunological responses is further explored in subsequent chapters with regard to modification of neutrophil behaviour by cytokines released by lymphocytes and monocytes.

During the course of work for this thesis, we became aware of an anti-inflammatory agent, tetrandrine, which is isolated from a traditional Chinese remedy for rheumatic diseases. Tetrandrine has been shown to be unique in its ability to retard, halt and even reverse the fibrotic lesions of silicosis. As the lesions of silicosis are derived mainly from host-mediated damage via the neutrophils and macrophages, we thought it likely that tetrandrine exerts effects on these phagocytic cells in the control of silicosis. Therefore, we carried out investigations to study the effect of this novel anti-inflammatory agent on various aspects of neutrophil functions. These are reported in the last two experimental chapters. The final chapter discusses all

the findings of the investigations in the thesis in the light of current understanding of the immunopathogenesis of oral diseases.

Immunobiology of the Neutrophil

Neutrophil physiology

The neutrophils are polymorphonuclear leukocytes characterised by their lobulated nuclei and granular cytoplasm. The multilobulated nucleus contributes to the extreme elasticity of the cell, allowing migration of the cell through such small openings as those found between junctions of endothelial cells (Marchesi and Florey, 1960). The numerous granules found in the cytoplasm are specialised lysosomes which have been classified as primary and secondary granules.

Neutrophils develop in the bone marrow from undifferentiated haematopoietic stem cells within a period of 11-12 days (Dancey et al, 1976). About 1.2×10^{11} mature neutrophils are released into the blood daily. The mature neutrophil is an endstage cell, with a brief half-life of about 3-4 hours (Issekutz & Movat, 1982).

In the blood, there are two subpools of neutrophils which are readily exchangeable: the circulating pool which forms the central axial stream, and the marginal

pool which consists of cells that move slowly along the vascular endothelium, occasionally adhering to the endothelial surface momentarily. For each circulating neutrophil, there is one in the marginal pool. In addition, for each peripheral mature neutrophil, there are 14 immature ones in the postmitotic compartment, and further reserves in the mitotic compartment of the bone marrow (Gallin, 1984).

The possibility of heterogeneity among circulating human neutrophils has been demonstrated in experiments showing that neutrophils can be divided into two populations based on their ability to form rosettes with IgG-coated erythrocytes. (Gallin, 1984). However, it is unknown whether this heterogeneity reflects true subpopulations of cells that originate from distinct stem cells or represents maturational differences within a common cell line (Gallin, 1984).

The functions of the neutrophil may be divided into two general groups. Firstly, these phagocytic cells remove pathogenic microorganisms, usually through an inflammatory reaction and specific immune reaction against the microorganism. Secondly, they clear damaged or senescent cells from tissue. To perform these functions, the neutrophils are equipped with wide-ranging special properties such as rapid locomotion,

phagocytosis and microbicidal mechanisms.

A. Locomotion:

i) Adherence

Increased adherence to the endothelium followed by diapedesis into the extravascular space appear to be important early events in the induction of the inflammatory response (Wilkinson, 1980). Time-lapse photography and photomicrography have shown that neutrophils adhere preferentially to vascular endothelium adjacent to a site of inflammation. This directed adherence is triggered by the binding of ligands on receptors on the neutrophils which elicit cellular changes that cause them to adhere (Hoover et al, 1978). Such physical alterations of the cells include ruffling of their plasma membrane, increases in surface area, and changes of shape from spherical to polarized. In addition, there is a decrease in negative net surface charge thus increasing their adherence to endothelial cells which also carry a negative surface charge (Harlan, 1985).

Once the neutrophil is effectively adherent to the vascular endothelium, it migrates into the extravascular space by the insinuation of pseudopods (projections of cytoplasm) into interendothelial cell junctions through a process of active cell motility.

The molecular basis of neutrophil adherence is currently being unfolded, with the discovery of several glycoproteins on surfaces of these cells which mediate adherence, and the production of monoclonal antibodies against these glycoproteins (Ho and Springer, 1983; Sanchez - Madrid et al, 1983). The first adherence glycoprotein called Mo1, is common to both neutrophils and monocytes. The second glycoprotein known as the LFA-1 (lymphocyte function-associated antigen), is found on lymphocytes (T cells, natural killer cells [NK cells], B cells), monocytes and neutrophils. The third antigen, called p150,95, is found on neutrophils and monocytes. These glycoproteins each contain a α chain of 150-177 kDa, and a β chain of 95 kDa, held together in a noncovalent linkage (Ho & Springer, 1983).

ii) Chemotaxis

Neutrophils show locomotor responses to environmental chemicals by responses known as chemotaxis and chemokinesis. Chemotaxis is a reaction by which a chemical attractant determines the direction of cellular locomotion, whereas chemokinesis is a reaction by which chemical substances determine the rate of cellular locomotion (Keller et al, 1977). Chemotactic gradients are important in directing cells to sources of inflammation or infection (Wilkinson, 1980).

A wide range of chemotactic factors have been

identified. The most important of these are microbial products and synthetic formyl peptide chemoattractants, as well as components of the complement system such as C5 fragments, C567 and a C3 fragment separate from C3a. (Ward et al, 1965; Schiffman et al, 1975). Other biologically relevant chemoattractants include chemotactic lymphokines (Cohen et al, 1979), a cell-derived chemotactic factor produced by the neutrophils (Spilberg et al, 1976), and neutrophil chemotactic factors contained in mast cells (Wasserman et al, 1977). In addition, products of arachidonic acid metabolism have also been shown to be chemoattractants, the most potent being leukotriene B₄ (Ford-Hutchinson et al, 1980).

The binding of chemoattractants to receptors results in increases in cytosolic free calcium alone or in concert with activation of protein kinase c (Lew et al, 1986; Sklar and Oates, 1985; Di Virgilio et al, 1984). In addition, there is initiation of phospholipase C-dependent hydrolysis of membrane phospholipids yielding inositol 1,4,5-triphosphate, an endogenous calcium ionophore and diacylglycerol, an activator of protein kinase C (Rostrosen & Gallin, 1987).

Movement of neutrophils is generated by the activity of microfilaments consisting of actin, myosin and associated proteins that mediate the contractions

and relaxations of the moving parts of the cell (Wilkinson, 1980). These microfilaments and associated proteins are most likely to be controlled by local changes in concentrations of calcium ions which results from the binding of chemotactic molecules to binding sites on the cell membrane of the neutrophil. It has also been suggested that these binding sites may have an indirect physical link to the submembranous microfilament network (Bourgignon & Singer, 1977).

The initial response of the neutrophil after binding of a chemotactic factor is polarization of the cell, the anterior aspect of the cell developing a broad lamellipodium, and the posterior a thin uropod which attaches to the substrate (Zigmond, 1977). Receptors which are normally randomly distributed over the unstimulated cell surface are found initially in the lamellipod and then move to the uropod in a phenomenon known as "tail lighting" (Walter et al, 1980). In addition, there is release of specific granule contents at the leading edges of the lamellipod associated with fusion of the granule membrane and the surface membrane (Gallin et al, 1978). Exposure of human neutrophils to degranulating agents enhances the number of chemotactic factor receptors on the cells, indicating that replacement of receptors on the surface of the lamellipod occurs partly via this membrane-granule fusion (Gallin

et al, 1978).

B. Phagocytosis:

The ability to bind and ingest microbes and foreign particles is a striking feature of the neutrophil. In carrying out this vital task, the neutrophil first recognises the intruder, usually with the aid of opsonins - proteins that bind specifically to the intruder as a signal for phagocytosis (Griffin, 1977). Well known opsonins include serum-complement component C3 and IgG antibodies. The C3b component appears to attach the microbe to the neutrophil and the Fc portion of the IgG induces ingestion of microbe (Scribner & Fahrney, 1976). When present together, immunoglobulin and complement opsonins facilitate ingestion at concentrations of either that would be ineffective if present alone.

After recognition and binding to the microbe, a number of intracellular processes are actively coordinated, culminating in the ingestion and killing of the microbe. Most obvious is the elaboration of pseudopods in the general direction of the particle. These pseudopods appear to result from local changes in the polymerization and cross linking of actin filaments (Azline & Raeven, 1974). The advancing pseudopods are

directed at each step of phagosome formation and fusion by sequential engagement of opsonin and receptor, a process called the "zipper hypothesis" (Wright, 1985).

Opsonin-receptor complexes on the neutrophil plasma membrane surface triggers the generation of intracellular second messengers activating the two major antimicrobial systems, O_2 -dependent and O_2 -independent which will be discussed in the next section.

C. Microbicidal systems:

Killing and digestion of ingested microorganisms is the ultimate aim of phagocytosis. Two main microbicidal systems exist in the neutrophil - the oxygen-independent system which consists of the lysosome enzymes, and the oxygen-dependent system consisting of the toxic oxygen radicals generated from the respiratory burst. The neutrophil is thus equipped to handle microbes in several environments including the absence of oxygen. The multiplicity of microbicidal systems is important to host defence as many microbes have developed effective countermeasures, eg production of enzymes, such as catalase and superoxide dismutase.

When phagocytosis is initiated, and following closure of the phagocytic vacuole, there is fusion of

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Killing and digestion of ingested microorganisms is the ultimate aim of phagocytosis. Two main microbicidal systems exist in the neutrophil - the oxygen-independent system which consists of the lysosome enzymes, and the oxygen-dependent system consisting of the toxic oxygen radicals generated from the respiratory burst. The neutrophil is thus equipped to handle microbes in several environments including the absence of oxygen. The multiplicity of microbicidal systems is important to host defence as many microbes have developed effective countermeasures, eg production of enzymes, such as catalase and superoxide dismutase.

When phagocytosis is initiated, and following closure of the phagocytic vacuole, there is fusion of

the invaginated plasma membrane with the granules of the phagocytic cell. This allows the granular contents to be discharged into the phagosome, a process known as degranulation, and the granule membrane constituents are translocated to the limiting membrane of the newly-formed phagolysosome (Hirsch & Cohn, 1960). In addition, there is activation of the respiratory burst with generation of highly toxic products of oxygen reduction (Babior, 1984). Phagocytosed particles are thus subjected to an environment of microbicidal systems, ie, the granule components and the toxic oxygen species which result from activation of the respiratory burst.

(i) Oxygen-independent antimicrobial systems

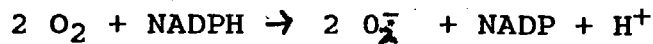
Human neutrophils contain two forms of lysosomal granules which have chemically distinct contents at the time of their formation in neutrophil precursors undergoing division in the bone marrow. The primary or azurophilic granule (peroxidase-positive), is formed during the promyelocyte stage and the secondary or specific granule during the myelocyte stage. Primary granules contain the important microbicidal enzyme myeloperoxidase (MPO), as well as a variety of acid hydrolases and neutral proteases that are involved in the digestion of ingested organisms or in the destruction of target cells. These compounds include lysozyme (muramidase), and cationic proteins (Root & Cohen, 1981;

Weiss et al, 1983), as well as a recently described family of homologous, low molecular weight (<3500 dalton) peptides, termed defensins because of their broad cytotoxic properties against bacteria, fungi, viruses and tumour cells (Ganz et al, 1985). Specific granules contain the antimicrobial substance lactoferrin, as well as approximately two-thirds of the cell's lysozyme.

(ii) Oxygen dependent antimicrobial systems

Membrane perturbation and exposure to soluble stimuli cause the neutrophil to initiate a respiratory burst which is also observed in other phagocytic cells such as monocytes, macrophages and eosinophils. There is a burst in oxygen consumption, as well as increase in glucose consumption via the hexose monophosphate shunt and production of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen (1O_2) [Babior, 1984]. Recent studies suggest that the oxidase enzyme involved in the reaction is a non-mitochondrial electron transport chain comprised of an FAD-requiring flavoprotein, an unusual b-cytochrome unique to phagocytes, cytochrome b_{558} , which is also designated cytochrome b_{245} , and possibly a quinone (Rotrosen and Gallin, 1987). Reduced pyridine nucleotide NADPH, generated by the hexose monophosphate shunt is the preferred electron donor used by the oxidase

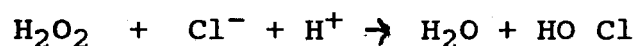
shown in the reaction below:



It is likely that translocation of the cytochrome occurs, with the density of specific granules, from an intracellular membrane component to the plasma membrane to complete the respiratory chain (Ohno et al, 1985).

Activation of the respiratory burst may be controlled by other factors which are not yet clearly elucidated. There is evidence that many independent transduction pathways exist and that changes in cytosolic calcium, intracellular pH, protein phosphorylation, transmethylation reactions and cell membrane attachment may play a role (Rostrosen & Gallin, 1987).

The microbicidal effects of superoxide generated by the respiratory burst probably results from its highly reactive products, H_2O_2 , $\cdot\text{OH}$, and $^1\text{O}_2$. It has been found that superoxide can cross cell membranes through anion channels or by penetration of the lipid layer (Lynch & Fridovich, 1978), and that H_2O_2 can also diffuse across cell membranes and gain access to intracellular organelles (Ohno & Gallin, 1985). By conversion to hypochlorous acid (HO Cl) in the presence of myeloperoxidase and chloride, the antimicrobial activity of H_2O_2 is greatly enhanced:



Chloride appears to be the main substrate although other halides can also be used. The bacterial cell wall is damaged by halide incorporation and hypochlorous acid reacts with chloride to form chlorine and potent long-lived N-chloro oxidants (Test et al, 1984).

Intracellular structures are protected from the effects of these free radicals by cytoplasmic enzymes, eg, superoxide dismutase converts the superoxide radical to hydrogen peroxide which is then transformed to water by catalase (Sbarra et al, 1972).

The neutrophil in host defence

The importance of the neutrophil in host defence is best evidenced by the severe infections seen in patients with neutrophil disorders, which may be classified into neutropenias and functional disorders. The most severe infections are suffered by patients with neutropenias. A neutrophil count of less than 1000 cells/mm^3 predisposes the patients to increased risk of infection and when there are fewer than 200 cells/mm^3 , the inflammatory response is essentially absent (Gallin, 1982). The causes of neutropenia are not completely understood but are related to depressed marrow production (idiopathic, drug-induced, tumour invasion, nutritional deficiency), peripheral destruction (antibody to

neutrophils, splenic trapping) or peripheral pooling in overwhelming infection (Roberts and Gallin 1983). Inherited types of neutropenia include Kostmann's syndrome which is severe and often fatal, more benign forms of chronic idiopathic neutropenia, the hair-cartilage-hypoplasia syndrome, Schwachman syndrome and neutropenias associated with other immune defects (Roberts and Gallin, 1983).

Functional disorders of the neutrophils also compromise host defences and affected patients suffer from recurrent infections, the severity of which depend on the degree of functional impairment of the cells as well as the effectiveness of compensatory mechanisms. Functional disorders may be classified into disorders of chemotaxis and disorders of phagocyte killing (Roberts & Gallin, 1983).

Disorders of chemotaxis may be divided into several categories. Firstly abnormal neutrophil adherence compromises the endothelial margination and tissue exudation of neutrophils, which are critical processes in the evolution of the inflammatory response. Hence recurrent or progressive infections are common in patients who suffer from the newly recognised genetic disorders of deficiency of M_0-1 , LFA-1 or p150,95-three structurally related "adhesive" surface glycopro-

teins, in which there are profound abnormalities of tissue leukocyte mobilisation, granulocyte-directed migration and hyperadherence (Anderson et al, 1984). Secondly, abnormal deformability of neutrophils such as seen in neonates is thought to hamper chemotaxis (Miller 1979). Thirdly, defective locomotion may be caused by abnormalities in structural and motor elements involved in neutrophil movements as seen in the Chediak-Higashi syndrome in which abnormal microtubular formation is related to abnormal functions of movement and degranulation (Roberts & Gallin, 1983). Patients with this syndrome suffer from recurrent pyogenic infections and many develop a fatal lymphomatous-like disease early in life, indicating that locomotive abnormalities in neutrophils compromise host defence severely.

Killing of ingested microorganisms represents the ultimate achievement of the neutrophil, and abnormalities of this function predispose affected patients to severe infections. Chronic granulomatous disease (CGD) is an example of such a cellular disorder where the phagocytes lack the capacity to convert oxygen to its microbicidal by-products, superoxide anion, hydrogen peroxide and hydroxyl radical. The recurrent abscesses of the skin and reticulo-endothelial system suffered by patients with CGD attest to the importance of an intact neutrophil microbicidal system for host defence (Roberts

& Gallin, 1983).

The types of infections suffered by patients with neutropenia and functional disorders of neutrophils are chiefly bacterial, such as Staphylococcal as well as fungal, such as Candidial, indicating that neutrophils are of prime importance in defence against such infections (Roberts & Gallin, 1983). However, the general protective role of the neutrophil also extends to defence against viruses (Klebanoff, 1980 & Siebens et al, 1979) as well as parasites (Czarnetzki, 1978). In addition, neutrophils are capable of destroying tumour cells, chiefly through antibody-dependent cellular cytotoxicity (ADCC) (Hokland & Berg, 1981). These latter activities have been demonstrated in-vitro, but their clinical relevance remains unclear.

The neutrophil in inflammation and tissue damage

Neutrophils play important roles in the initiation, development and regulation of the acute inflammatory response which is vital to host defence. However, the effects of the neutrophils in host defence is likened to that of the proverbial two-edged sword - in the process of killing microorganisms, the stimulated neutrophils liberate lysosomal enzymes, toxic oxygen radicals and inflammatory mediators which damage surrounding tissues.

Early in the inflammatory response, the greatly increased cell adhesiveness observed after exposure to chemotactic factors may result from the release of acidic proteins found in secondary granules, which neutralises the neutrophil surface charge, and enables contact with the endothelial cells (Boxer et al, 1982). The secretion of proteases such as elastase and collagenase, together with synergistic effects from oxygen metabolites causes loosening of the endothelial cell junctions and disruption of the basement membrane, thus allowing ease of emigration of the neutrophils into the tissue (Harlan et al, 1980; Taubman & Cogen, 1975).

The secretion of enzymes also provides a mechanism by which neutrophils may amplify the inflammatory response and promote their accelerated accumulation. This amplification effect may be mediated both by the generation of vasoreactive mediators that enhance local blood flow and increase vascular permeability, and by generation of chemotactic factors. Various proteases from neutrophil granules are able to cleave protein substrate in plasma, leading to the production of kinins and anaphylatoxins (Ward, 1985). Furthermore, granule-derived mediators released by stimulated neutrophils have been known to cause the release of vasoactive amines from mast cells and platelets (Kelly et al, 1971;

Hallgren & Venge, 1976).

In addition to their important role in the initiation and development of inflammation, neutrophil granule components which are liberated during cell stimulation or lysis can also be related to tissue damaging effects. The enzymes most important in this regard are the proteases, of which elastase is the most abundant. Other proteases implicated in tissue destruction are cathepsin S, collagenase and gelatinase (Ward, 1986). These proteases cause detachment of cells to their underlying matrix attachment and can hydrolyse a variety of connective tissue matrix substances such as collagen, basement membrane, glycoaminoglycans etc.

In addition to granule mediators of inflammation, oxygen metabolites produced by the neutrophils also have important roles in the inflammatory response and tissue damage.

Oxygen radicals such as H_2O_2 , $\cdot OH$ and 1O_2 generated during the respiratory burst in the neutrophil are extremely reactive and can initiate a variety of chemical reactions in both cellular and noncellular targets. Damage or destruction of several cell types such as endothelial, smooth muscle, hepatocytes, lymphocytes and myocytes have been described (Weiss,

1983). These changes result from diverse chemical reactions such as cross-linking of membrane and intracellular proteins, peroxidative changes in membrane lipids and DNA damage. In addition, oxygen radicals are able to react with substrates such as vascular basement membrane resulting in greatly augmented hydrolysis by leukocytic proteases (Fligiel et al, 1984). There is also recent evidence that oxygen products (probably HOCl), can activate two latent proteases of the neutrophil, collagenase and gelatinase (Weiss et al, 1985). Other effects of the oxygen radicals which are of relevance to their tissue damaging potential include oxidative activation of alpha₁-antiproteinases (the main regulator of leukocytic proteases), generation of chemotactic lipid from arachidonic acid and activation of the complement system by unknown mechanisms (Ward, 1986). Thus the oxygen radicals generated by the neutrophils upon stimulation are extremely toxic, not only because of their intrinsic reactivity, but also through their potentiating effects on other mechanisms of tissue damage.

Beside the granule components and the oxygen metabolites generated during stimulation, other inflammatory mediators are generated from plasma membrane lipid metabolism. Like many other inflammatory cells, neutrophils are equipped with the two principal pathways

for oxidative metabolism of arachidonic acid, namely, the cyclo-oxygenase pathway which produces prostaglandins (PGs), and the 5-lipoxygenase pathway which produces leukotrienes (LTs). The prostaglandins and leukotrienes are potent inflammatory mediators, indicating that through their actions, neutrophils can modulate the inflammatory process.

Of the various cyclooxygenase products formed during inflammation, PGE_2 and prostacyclin are the most important. These products are both potent vasodilator and hyperalgesic agents and are thought to contribute to the erythema, oedema and pain which are characteristic of the inflammatory response (Salmon & Higgs, 1987).

Of the leukotrienes, LTB_4 is one of the most potent inflammatory mediators. The neutrophil is an important source of LTB_4 and the release of this powerful chemoattractant causes the recruitment of other neutrophils into the inflammatory site, thus amplifying the response (Higgs et al, 1979). In addition, lipoxygenase products may play a role in the influx of calcium when neutrophils are stimulated with chemoattractant (Volpi et al, 1980). In particular, LTB_4 is thought to be an endogenous calcium ionophore in the leukocyte and may occupy a pivotal role in the control of calcium-dependent functions such as movement and degranulation (Serhan et

al, 1982).

The neutrophil in immunoregulation

There is increasing recognition that neutrophils do not only respond to inflammatory signals but are able to influence the behaviour of other inflammatory cells as well. The first evidence of this was reported in 1968 by Ward who described a chemotactic factor derived from the granules of rabbit heterophils that was specific for monocytes (Ward, 1968). Since then several reports have indicated that a variety of neutrophil-derived factors can modulate responses of several inflammatory cell types such as monocytes, lymphocytes and eosinophils. In addition, factors that influence the behaviour of neutrophils themselves have been reported (Luciak et al, 1986; Ishibashi & Yamashita, 1981).

The chemotactic factor specific for monocytes described by Ward has been confirmed in studies with human neutrophils (Wright & Greenwald, 1979). In addition, these studies also showed that transformation of blood monocytes to macrophages was stimulated by secondary granule protein from human neutrophils (Wright & Greenwald, 1979). More recently, a phagocytosis-stimulating factor derived from guinea pig neutrophils has been shown to enhance the initial rate of phagocytosis of serum-opsonized zymosan particles by macrophages.

(Ishibashi & Yamashita, 1987).

The immune responsiveness of lymphocytes has also been shown to be modulated by neutrophil-derived factors. Elastase and Cathepsin G from the primary granules may stimulate lymphocyte proliferation in-vitro as B-cell mitogens (Vischer et al, 1976). In addition, lysosomal enzymes have also been shown to enhance immunoglobulin synthesis of human lymphocytes (Yamasaki & Ziff, 1977). Also, enhancement of both thymocyte stimulation by phytohaemagglutinin and mitogenic stimulation of mouse splenocytes was effected by a neutral protease recovered from the extracellular media of cultured mouse neutrophils (Lamster et al, 1979; Yoshinaga et al, 1980). More recent evidence of neutrophil-derived mediators on the enhancement of lymphocyte function includes the "lymphocyte-activation factor", originating from neutrophil granules (Harris, 1982), as well as the neutral protease similar to Cathepsin G, which stimulated lymphocytes in-vitro (Panush, 1983). Also, a helper T-cell enhancing factor which induced both proliferation and differentiation of lymphocyte population was isolated by Fitzgerald and coworkers (Fitzgerald et al, 1983).

Besides lymphocyte-enhancing mediators, neutrophils also release an eosinophil chemotactic factor which

appears to be derived from the plasma membrane (Czar-netzki, 1978; Frickhofen & Kowig, 1979).

There is also increasing evidence to show that in addition to the release of factors which affect other inflammatory cells, neutrophils also release mediators that regulate their own activities. Lactoferrin, a component of neutrophil secondary granules, acts as a negative feedback regulator of neutrophil precursor cell proliferation and differentiation in the bone marrow (Broxmeyer et al, 1978; Perlus et al, 1979). This effect appears to be achieved by interfering with the release of colony-stimulating factor (CSF) from marrow mononuclear cells (Broxmeyer et al, 1978). Thus, removal of mature neutrophils from storage pools as they enter the circulation may stimulate myelopoiesis by the removal of an inhibitory product of these cells, such as lactoferrin. The importance of negative feedback regulation in myelopoiesis is supported by observations made in human cyclic neutropenia (Wright et al, 1981).

A heat-stable phagocytosis-stimulating factor (PSF) generated from neutrophils during phagocytosis has been described by Ishibashi & Yamashita (1981,1982). This has been recently purified to homogeneity (Ishibashi & Yamashita, 1982) and its precursor has been identified in the granule fraction (Ishibashi & Yamashita, 1985).

Recently, granule products of neutrophils have been shown to decrease the expression of Fc receptors on other neutrophils but the clinical significance of this is not clear (Luciak et al, 1986).

The Neutrophil in Oral Diseases

Neutrophils constitute the largest proportion of phagocytic cells found in the oral secretions, yet their roles in the oral cavity have not been well defined. Only the function of defence by these cells have been emphasised; this is evidenced by the numerous reports documenting severe oral ulcerations and periodontal breakdown in patients with neutropenias and diverse neutrophil dysfunctions (Arnold & Hoffman, 1979; Cohen & Morris, 1961; Tempel et al, 1972; Quie, 1969). However, other possible roles of neutrophils in the oral cavity such as in inflammation and tissue breakdown as well as in immunoregulation have not been well recognised.

In this section, the source of neutrophils in the oral cavity as well as the role of the neutrophils in 3 dental diseases, namely periodontal disease, dental caries and dental pulp inflammation, are reviewed with an attempt to place the respective roles of these phagocytic cells into perspective.

A. Source of neutrophils in the oral cavity

Like other mucosa-lined cavities challenged by a variety of microbes, the oral cavity is continually bathed with fluids containing large numbers of neutrophils. Microscopic examination of saliva usually shows the presence of these cells, although they may be absent from saliva collected directly from the ducts and very few are present in saliva from infants before eruption of teeth, or from edentulous patients (Wright, 1964). This indicates that the major site of entrance of neutrophils in the oral cavity is via the gingival sulcus, where they constitute 95-97% of the cells in crevicular fluid (Loe, 1961; Attstrom, 1970). Even healthy gingivae allow some leukocytes to enter the mouth, the numbers increasing four to five-fold between waking and midday (Schiott & Loe, 1970). With the development of gingival inflammation, the number of leukocytes migrating into the sulcus increases significantly (Kowashi et al, 1980).

Electron microscopy has shown that a few neutrophils can generally be seen within the intracellular spaces of the junctional epithelium of the gingiva even in clinically health gingival tissue (Schroeder, 1970). The migration is probably a response

to the presence of bacterial or host-activated chemotactic substances (Temple et al, 1969; Wennstrom et al, 1980). In addition, free lysosomal bodies, presumably of neutrophil origin, have been demonstrated in the intracellular spaces of the junctional endothelium as well as in the connective tissue adjacent to this epithelium (Freedman et al, 1968).

Most of the crevicular neutrophils are found to be viable as assessed by trypan blue exclusion (Skapski & Lehner, 1976). In addition, they have been shown to have phagocytic activity (Scully, 1980; Wilton, 1982) and respond to bacterial stimuli with an increase in metabolic activity (Wilton et al, 1977).

The role of the neutrophil in dental caries

Dental caries is a multi-factorial disease involving interaction between the dental hard tissues, dietary sugars and cariogenic bacteria. Current concepts of the aetiology of dental caries are based on Miller's chemicoparasitic theory which states that dissolution of hard tooth substance occurs from the organic acids produced by the cariogenic bacteria acting on fermentable sugars in the mouth.

The importance of bacteria in the aetiology of dental caries is demonstrated by the classical experi-

ments of Orland et al (1954) which showed that germ-free animals did not develop caries in spite of being fed large amounts of sucrose. Subsequently, several organisms capable of inducing caries in animals were identified. Of crucial importance is *Streptococcus mutans*, which, due to its great acidogenic potential and its ability to form large quantities of extracellular dextrans for attachment to the smooth tooth surface, is one of the most cariogenic organisms ever isolated (Hamada & Slade, 1980). Other organisms implicated in dental caries include lactobacilli (Fitzgerald, 1968) which are probably important only in caries of pit and fissures, or as a secondary invader in cavities already initiated by other organisms, since it lacks the ability to attach to smooth surfaces. *Actinomyces viscosus* has been suggested as an important cariogenic organism in caries of the root surface (van der Hoeven et al, 1972).

Strains of *Streptococcus mutans* have been divided into seven serotypes (a,b,c,d,e,f,g), with serotype c being the most commonly isolated serotype (Duany et al, 1972; Bratthall, 1970; Grenier et al, 1973; Perch et al, 1974; Shklair & Keene, 1974; Hamada et al, 1976). Reasons for the propensity of this serotype to colonise the oral cavity are not clear. Previous investigators have mainly suggested microbiological virulence factors, and the possibility of evasion of host-defence factors

by this serotype has been largely ignored.

As the hard dental tissues are immunologically inactive, host defence mechanisms involved in dental caries are centred mainly around the prevention of attachment and removal of cariogenic microorganisms. In this regard, the neutrophils are of prime importance as they constitute over 97% of the cells emigrating from the gingival sulcus and are the first cells to encounter any potential cariogenic organism on the tooth surface. The neutrophils are probably most effective at the earliest stage of plaque formation when few bacteria are present. Therefore, any organism that is capable of circumventing these defence cells increases its chance of attaching to the tooth surface, where it can multiply and cause demineralization.

Interactions of neutrophils and cariogenic bacteria are most likely to be direct. This is because, although immunoglobulins may facilitate neutrophil activities against *Streptococcus mutans*, their concentrations in the gingival sulcus is small and probably of minimal clinical relevance (Clagett & Page, 1978; Hsu & Cole, 1985). In addition, antibody directed against *Streptococcus mutans* is practically non-existent following natural infection with this bacteria (Hamada & Slade, 1980; McGhee & Michalek, 1981).

The role of the neutrophil in pulp diseases

The dental pulp is typical connective tissue with fibres and cells embedded in ground substance matrix. Of great clinical relevance is the fact that the dental pulp is almost completely enclosed within hard dental structures except at tiny openings at the ends of the roots. Hence, the increase in hydrostatic pressure resulting from acute inflammation within such a confined space is usually associated with severe pain (Seltzer & Bender, 1984). In addition, extensive inflammation often results in necrosis of the entire pulp (Langeland, 1987).

Like elsewhere in the body, the neutrophils in the dental pulp may have roles in defence, inflammation and tissue damage as well as immunoregulation. The defence roles of the neutrophils are well demonstrated in histological sections of dental pulps exposed to caries, where infiltrations of neutrophils are always evident, with many of the neutrophils appearing to have phagocytosed microorganisms (Seltzer et al, 1961).

The role of the neutrophil in pulpal inflammation induced by caries has also been well documented. Histochemical studies have indicated that during the acute phases of caries-induced pulpitis, collagen fibres

become fragmented and protein-carbohydrate complexes become markedly degraded (Zerlotti, 1969). Most of the tissue breakdown has been attributed to lysosomal enzymes released by the neutrophils and macrophages during stimulation and cell death as bacterial invasion of the pulp occurs only in very late stages of the disease (Zerlotti, 1969; Langeland, 1987). However, this role of the neutrophil in tissue damage has been stressed previously only in studies in caries-induced pulpitis; in other types of pulpal inflammation such as those induced by physical trauma (eg, through the dental drill) and irritant medicaments, the damage has been mainly attributed to the direct irritating effect of these factors on the connective tissue cells, eg, odontoblasts. (Langeland, 1961; Nyborg & Brannstrom, 1968; Seltzer & Bender, 1984; Brannstrom and Nyborg, 1960). The neutrophil, being one of the first cells to respond to noxious stimuli such as physical trauma and chemical irritants, may do so with degranulation of its lysosomal contents as well as production of toxic oxygen free-radicals which can contribute to further tissue damage. However, the various medicaments used for the treatment of pulp conditions have only been minimally assessed with regard to their effects on neutrophils. Doblecki et al (1980), showed that zinc oxide-eugenol cements increased neutrophil migration and

these cements have been implicated in non-specific inflammation (Campbell et al, 1978). However, the effects of other pulp medicaments on neutrophils have not been previously reported. It is likely that the medicaments which suppress the neutrophils will cause less inflammation of the pulp than those which stimulate them.

The role of the neutrophil in immunoregulation of the pulp has hardly been investigated. The response of the pulp to many types of sustained oral stimuli is usually a short period of acute inflammation followed by chronic inflammation, which may be interspersed with periods of exacerbations of acute inflammation (Langeland, 1987). In the acute phases, the neutrophils are present in great numbers and it is likely that immunomodulatory factors are released by them which control the influx and behaviour of other inflammatory cells.

The role of the neutrophil in periodontal diseases

Introduction

Periodontal diseases, responsible for a large proportion of tooth loss in adults, are a group of poorly understood inflammatory conditions involving tooth supporting tissues, ie, gingival tissues, periodontal ligament fibre, alveolar bone and cementum. The disease occurs in varying grades of severity, with

minimal disease confining to the gingiva alone (gingivitis), and extensive disease destroying other periodontal structures (periodontitis).

The major aetiologic agent in the pathogenesis of periodontal diseases is microbial dental plaque. Subgingival plaques, situated below the gingival margin, are especially relevant to periodontal diseases and the major Gram-negative organisms implicated in periodontal diseases collectively include: Acidaminococcus, Bacteroides, Capnocytophaga, ~~Campylobacter~~, Fusobacterium, Haemophilus, Leptotrichia, Propionibacterium, Spirochaetes, Veillonella and Vibrio. Gram-positive organisms are represented by Actinomyces, Arachnia, Eubacterium, Lactobacillus, Peptococcus, Peptostreptococcus, Propionibacterium and Streptococcus (Socransky, 1977; Van Palenstein Halderman, 1981). Local variables such as stage of lesion development as well as the type of periodontal disease, may affect the qualitative and quantitative nature of the microbiota of these plaques. Several putative periodontopathic organisms have been identified among the general commensal bacteria in plaque. However, it has not been proven unequivocally that certain organisms are the definite pathogens (Genco & Mergenhagen, 1980). Moreover, in any single lesion of periodontal disease, there is a continually changing microbial flora so that organisms that are important in

the early development of a lesion may not be relevant in the later stages (Taichman et al, 1984)

Similarly, the inflammatory changes that are observed in the periodontal lesions result from many dynamic processes involving the complex interplay of both protective and destructive effector mechanisms (Genco & Mergenhagen, 1980; Page & Schroeder, 1982). Neutrophils are present in all stages of the development of periodontal diseases and are likely to be an important determinant in the pathogenesis of inflammation (Page & Schroeder, 1976). As in most other types of inflammatory diseases, the roles of the neutrophils in periodontal diseases involve defence, inflammation and tissue damage as well as immunoregulation.

Role of neutrophils in defence

The defence role of the neutrophils are affected by peculiar conditions that are present in the gingival crevice. The dental plaque that confronts them is a relatively thick mass consisting of aggregates of bacteria. Therefore, although attracted to the infected sites, the neutrophils are usually unable to phagocytose these dense masses of bacteria once plaque has begun to form (Taichman et al, 1984). However, in the earliest stages of infection, before plaque development has occurred, ie, when only small numbers of bacteria are

present, neutrophils probably play an important defence role in the gingival crevice. The corollary of this concept is that organisms that are capable of evading the neutrophils will probably be more likely to be successful in the colonisation of the gingival crevice.

That neutrophils constitute a significant element of defence in the gingival crevice is evidenced firstly by the fact that patients with neutrophil abnormalities have extremely severe periodontal destruction and mucosal ulceration. Neutropenic states have been associated with rapidly progressive forms of periodontal disease (Page & Schroeder, 1982). Periodontal manifestations have been reported in patients with agranulocytosis (Davey & Korchak, 1969), chronic benign granulocytopenia in childhood (Reichart & Dornow, 1978), and cyclic neutropenia (Cohen & Morris, 1961; Degnan & Perlov, 1973). In addition, patients with depressed neutrophil function are also reported to have severe periodontal lesions. These disorders include the Chediak-Higashi syndrome (Temple et al, 1972), chronic granulomatous disease (Charon et al, 1985), and abnormalities of neutrophil surface glycoproteins (Anderson et al, 1984).

Secondly, observations that certain oral pathogens such as *Bacteroides* and *Actinobacillus* (*Haemophilus*), with leuko-aggressive and leukotoxic properties against

neutrophils, are able to establish and maintain themselves in the periodontal pocket and even to invade gingival connective tissues, attest to the fact that being able to circumvent these important phagocytes contribute to their successful colonisation (Genco & Slots, 1984).

Thirdly, studies of neutrophil functions in localised juvenile periodontitis strongly suggest that neutrophil defects in these patients contribute to the colonisation and growth of the bacteria commonly associated with this condition, namely, *Haemophilus* (*Actinobacillus*) *actinomycetemcomitans*, *Capnocytophaga*, and *Eikenella corrodens* (Genco & Slots, 1984). In a large proportion of affected patients, chemotaxis was found to be depressed in neutrophils from peripheral blood (Van Dyke et al, 1985; Sandholm, 1985), as well as in neutrophils from the gingival crevice (Singh et al, 1984; Newman & Addison, 1982). Phagocytosis was also found to be depressed in these patients (Suzuki et al, 1984) although other neutrophil functions appeared normal (Ellegaard et al, 1984). In further studies, Van Dyke et al (1985) showed that the defect is cellular and associated with defects in FMLP and C5a binding. Thus, in localised juvenile periodontitis, depressed neutrophil functions enable the colonisation and growth of aggressive, opportunistic organisms which are not

usually found in other types of periodontal inflammation (Mashimo et al, 1983).

Role of neutrophils in inflammation and injury

The local influx of neutrophils into the gingival area is also associated with inflammation and destruction of host tissues. Stimulated neutrophils produce toxic oxygen radicals and lysosomal enzymes which not only destroy tissue but may enhance other inflammatory cells. Neutrophils buttressed directly against the dense aggregates of plaque bacteria are unable to phagocytose the bacteria but are stimulated to degranulate (Freedman et al, 1968; Schroeder, 1973). Evidence of this is provided by studies showing that there are elevated levels of collagenase, elastase, cathepsin and prostaglandin in both tissue and crevicular fluids (Cimasoni, 1983). It is well accepted that neutrophils collagenase and elastase may serve as important mediators of connective tissue destruction in inflamed gingiva (Narayan & Page, 1983).

In addition, it has been shown in experimental investigations in animals that neutrophils contribute to periodontal injury. Rabbits injected with intradermal suspensions of dental plaque showed considerable tissue damage and extensive inflammation characterised by dense neutrophil infiltration, and local release of lysosomal

products (Freedman et al, 1976). In contrast, these signs were not observed in leukopenic rabbits (Freedman et al, 1976; Taichman et al, 1966). Other workers have also shown that in dogs, gingival inflammation may be reduced, at least on a short term basis, by rendering them neutropenic (Kahnberg et al, 1976; Rylander et al, 1975). In addition, neutrophil-dependent models of inflammation, eg, Arthus -type reactions, can be induced in healthy gingival tissues, resulting in pathological states which bear striking resemblance to those occurring in the natural condition (Ranney & Zanders, 1970). Neutrophils are also important mediators of inflammation in the accelerated forms of periodontitis induced by ligatures (Page & Schroeder, 1982). In these animal experiments, the depletion of neutrophils with gold salts resulted in significantly less neutrophil exudation and periodontal destruction (Taichman et al, 1984).

Although there are now several investigations describing the roles of the neutrophil in defence and tissue injury, there is a paucity of information relating to the role of neutrophils in the immunoregulation of periodontal disease. This is not surprising as the latter role of the neutrophil in general disease has only been recently emphasised.

Regulation and Control of Neutrophils

Biological Control by Lymphokines and Monokines

The inflammatory response represents a complex series of events which are controlled by various mediators released by the inflammatory cells. Thus, the neutrophils which are capable of producing various cytokines involved in immunoregulation are themselves subject to influence from other factors released by other inflammatory cells. Of importance are the cytokines generated by lymphocytes and monocytes-macrophages. Large numbers of these lymphokines and monokines have been described but only a few have been well characterised. These include γ -interferons, interleukin -2 and tumour necrosis factor- β which are secreted by the lymphocytes and interleukin-1 and tumour necrosis factor- α secreted by the monocytes-macrophages.

i) Interferons

The interferons (IFNs) are characterised by their ability to induce cells to make new RNA and protein and to prevent viral replication (Friedman & Vogel, 1983). IFNs are a heterogenous family of proteins and may be classified into Type I or viral IFN which is induced by

virus infection or bacterial stimulation, and Type II, which is immune IFN induced by specific antigens, mitogens or other stimuli (Trinchieri & Perussia, 1985). Immune IFN or IFN- γ is the main type produced by stimulated T-lymphocytes while IFN- α and IFN- β are the predominant forms of viral IFN produced by leukocytes and fibroblasts. Although IFN- γ shares many characteristics and functions with IFN- α and IFN- β , much smaller concentrations are required for it to mediate the same effect. In addition, other functions are mediated only by IFN- γ (Trinchieri & Perussia, 1985).

The action of IFNs are exerted by their binding to specific membrane receptors on target cells which are then induced to undergo a series of metabolic modifications involving de novo synthesis of RNA and polypeptides. Receptors for IFNs are found on various cell types, indicating that it has wide-ranging immunoregulatory properties.

IFN induces the appearance of new surface markers or receptors associated with differentiation, and in the case of T and possible NK cells, also enhances proliferation (Wong et al, 1983). With regard to humoral B-cell responses, IFN- γ potentiates immunoglobulin secretion when added late during an immune response in vitro, and

can substitute for a late helper factor, often referred to as T cell replacing factor (TRF) (Leibson et al, 1984).

Monocyte-macrophage functions are also affected by IFN- γ . It appears to be one of the macrophage-activating factors (MAF) and induces or enhances the cytotoxicity of human monocytes against tumour targets (Le et al, 1983). Moreover, it also induces activation of various antiparasitic mechanisms in macrophages (Nathan et al, 1983). However, although IFN- γ acts synergistically with many lymphokines, it can also antagonise some of these, such as the inhibition of the collagen synthesis, antagonising the action of interleukin-1 (Rosenbloom et al, 1986).

The effects of IFNs on neutrophils are only beginning to be understood. Generally, stimulation of neutrophil function has been described. These include enhancement of IFN of in-vitro phagocytosis (Melby et al, 1982; Jarstrand & Einhorn, 1983), hexose monophosphate shunt activity (Ferrante & Rencis, 1984) as well as antibody-dependent cell-mediated cytotoxicity (Hokland & Berg, 1981). In addition, stimulation of nitroblue tetrazolium dye reduction by IFN has also been observed in vitro (Jarstrand & Einhorn, 1983; Pak et al, 1980), and in vivo (Einhorn & Jarstrand, 1984).

ii) Interleukin 2

Interleukin 2 (IL-2), a glycoprotein of 15,000 MW secreted by T lymphocytes, is also known as T-cell growth factor, as it functions as an obligatory signal for T-cell growth (Bendtzen, 1983). IL-2 receptors are expressed on T-cells after being given an initial signal by macrophage-processed antigen, alloantigen or lectin (Smith et al, 1980). Once these receptors are expressed, the presence of IL-2 is the sole limiting factor for proliferation and clonal expansion of the activated T-cells which may be helper, suppressor or killer T-cells (Bendtzen, 1983).

Although the effects of IL-2 on lymphocytes are well investigated, its effects on neutrophils have not been previously reported. It is likely that a cytokine with such important functions on lymphocytes, has effects on other inflammatory cells such as the neutrophils.

iii) Interleukin 1.

Interleukin 1 (IL-1), a polypeptide of molecular weight 17,000 daltons, has several acronyms which describe its wide range of actions in inflammation and immunoregulation. These include lymphocyte activating factor, mitogenic protein, B-cell activating factor, B-

cell differentiating factor, T-cell replacing factor, endogenous or leukocyte pyrogen, and leukocyte endogenous mediator (Billiau et al, 1985; Dinarello & Meir, 1986). Originally described as released from macrophages, two structurally related IL-1, alpha and beta, are now found to be released by many different cell types upon appropriate stimulation. These cells include endothelial cells, dendritic cells, Langerhans cells, fibroblasts and neutrophils, and the stimuli for triggering IL-1 release include lipopolysaccharide and the phorbol esters (Dinarello & Meir, 1986). With reference to dental disease, IL-1 has been found in gingival fluid (Mergenhausen, 1984) and is likely to play an important role in periodontal disease.

IL-1 affects several types of cells involved in immune responses including T-cells, B-cells, macrophages and neutrophils. The mitogenic effect on thymocytes was recognised earliest and remains the reference assay for IL-1. It is now known that this effect of IL-1 is in fact mediated by its ability to induce IL-2 production by the T-cells (Larsson et al, 1980).

IL-1 often augments lymphocyte activation primarily by inducing the synthesis of other lymphokines and the activation of the resting T-cell (Dinarello & Meir, 1987). Recently, IL-1 was discovered to act as a

cofactor with colony-stimulating factors in the promotion of bone marrow precursors, after suppression by cytotoxic drugs (Dinarello & Meir, 1987). IL-1 is also thought to play an important role in the pathogenesis of rheumatoid arthritis by inducing protease and bone resorption (Krane et al, 1985)

Neutrophils are also affected by IL-1. Subcutaneous injection of IL-1 leads to margination and extravascular infiltration of neutrophils (Granstein et al, 1985). In vitro studies indicate that IL-1 is a chemotactic attractant for neutrophils (Sauder et al, 1984), as well as increasing their glucose metabolism, and the reduction of nitroblue tetrazolium (Luger et al, 1983).

iv) Tumour necrosis factor- α

Tumour necrosis factor- α (TNF- α) is a polypeptide hormone of approximately 17 kilodaltons secreted in abundance by endotoxin-activated macrophages (Tracey et al, 1986). Its name was first given to describe the cytotoxicity of selected tumour-cell lines seen upon its administration. It is also known as cachetin because of the profound wasting seen in animals treated with it.

Although TNF- α has widespread effects on many tissues, its mechanisms of action are not well inves-

tigated. Its biochemical effects include suppression of biosynthesis of several adipocyte-specific mRNA molecules and prevention of pre-adipocyte differentiation (Torti et al, 1985). It also alters the haemostatic properties of the vascular endothelium, inducing the production of procoagulant activity and inhibiting the expression of thrombomodulin at the cell surface (Nawroth & Stern, 1986). These effects may enhance thrombi formation, leading to disseminated intravascular coagulation and to occlusion of tumour vessels (Beutler & Cerami, 1987).

TNF- α is now considered a mediator of general inflammation. It is an endogenous pyrogen, capable of inducing fever, both through a direct effect on hypothalamic neurons and through the peripheral induction of interleukin-1 (Dinarello et al, 1986). In addition, recent data suggests that IL-1 and TNF- α act synergistically in a variety of biologic responses, including tumour necrosis (Dinarello & Meir, 1987). TNF- α also exhibits osteoclast activating factor activity and is capable of stimulating synovial-cell production of prostaglandin E₂ and collagenase (Beutler & Cerami, 1987). It is thought that TNF- α mediates the effects of endotoxin and is directly responsible for the physiological changes in Gram-negative sepsis (Beutler & Cerami, 1987).

TNF- α has been reported to have stimulatory effects on neutrophils, enhancing their adhesion to endothelial-cell surfaces and increasing their phagocytic activity (Gamble et al, 1985; Shalaby et al, 1985).

Recently, it has been reported that TNF- α induces the in vitro differentiation of certain myeloid cell lines (Takeda et al, 1986). As well, it can induce GM-CSF production by a variety of cell types (Munker et al, 1986).

v) Tumour necrosis factor- β (TNF- β , lymphotoxin)

TNF- β or lymphotoxin is a lymphokine which specifically inhibits tumour cell growth in vivo as well as in in vitro (Papermaster et al, 1979; Gately et al, 1976). It suppresses the transformation of cells induced by chemical carcinogens or ultraviolet radiation (Evans et al, 1977; Evans & Di Paolo, 1981). Lymphotoxin has now been purified and its molecular weight has been determined to be approximately 20,000 daltons.

Since its purification, there has been interest on possible effects of lymphotoxin on various inflammatory cells (Dinarello & Meir, 1987). However, little information is available on the effects of this lymphokine on the neutrophils.

2. Chemical Control by Anti-Inflammatory Drugs

Besides being regulated by the cytokines released by other inflammatory cells, neutrophils are also influenced by many drugs. Of importance are the anti-inflammatory agents used to control a variety of acute and chronic inflammatory conditions. These include the corticosteroids, the non-steroidal anti-inflammatory drugs (NSAIDs), antimalarials, gold compounds, D-penicillamine and cyclosporin. In this section, the effects of these drugs on neutrophils will be discussed.

i) Corticosteroids

It has been known for a long time that treatment with glucocorticoids increases susceptibility to infections. This prompted many investigations into their effects on neutrophil numbers and functions (Axelrod, 1976). It is now known that corticosteroids exert their anti-inflammatory influence through suppression of peroxidation of arachidonic acid which generates the inflammatory molecules, prostaglandins and leukotrienes (Higgs, 1984). As these molecules have potent effects on neutrophil functions, it is likely that alterations of neutrophil functions by corticosteroids occur. In addition, it is also possible that corticosteroids inhibit the neutrophil directly through a membrane stabilising effect (Weissman, 1972).

Several investigators have reported that the adherence of neutrophils are decreased with steroids both in-vitro (MacGregor, 1974; Clark et al, 1979), and in vivo (McGillen & Phair, 1979). Locomotion of neutrophils are also impaired (Ward, 1966; Spisani et al, 1978) and their numbers are reduced in inflammatory exudates when dexamethasone is administered (Higgs et al, 1979). Migration into skin windows or inflammatory exudates is also depressed by steroid therapy (Dale et al, 1974).

In addition, hydrocortisone in high concentrations reduces the oxidative burst which accompanies phagocytosis and interferes with killing of *Staphylococcus aureus* (Mandell et al, 1970). Also, neutrophils from patients receiving glucocorticoids demonstrate inhibited reduction of nitroblue tetrazolium (Chretien & Guragusi, 1972).

ii) Non-Steroidal Anti-Inflammatory drugs (NSAIDs)

The NSAIDs inhibit the cyclo-oxygenase enzyme which is involved in the synthesis of prostaglandins from arachidonic acid (Vane, 1978). These drugs include aspirin as well as the newer NSAIDs, such as piroxicam, naproxen, sulindac and ibuprofen. Although the prostaglandins possess inflammatory properties, their inhibition by the NSAIDs may not be the only anti-

inflammatory mechanism of the drugs. This is shown by the fact that not all NSAIDs inhibit the cyclo-oxygenase enzyme effectively, yet their clinical effectiveness is not diminished (Abramson, 1985). In addition, there is often a disparity between the higher concentrations of NSAIDs required for anti-inflammation compared to the lesser concentrations necessary to inhibit prostaglandin synthesis.

Recently there has been great interest in the effects of NSAIDs on the neutrophil, a cell which plays a central role in arthritis, an inflammatory condition which may be controlled by NSAIDs, even though the main phlogistic products of the neutrophils do not derive via the cyclo-oxygenase pathway.

The effects of NSAIDs on neutrophils have been noted both in-vitro and in vivo. In-vitro studies on the locomotion of neutrophils indicated that ibuprofen, naproxen, sodium salicylate, ketoprofen, aspirin and indomethacin all have inhibitory effects on neutrophil chemotaxis, with ibuprofen giving the most profound response at equal concentrations (Spisani et al, 1978).

Other in-vitro studies of neutrophils pre-incubated with ibuprofen and piroxicam and then stimulated with FMLP indicated that these drugs depressed aggregation in

a dose-dependent manner (Abramson, 1984). However, other neutrophil responses varied among the NSAIDs. For example, ibuprofen had no effect on superoxide generation but reduced the amount of lysosomal release. In contrast, piroxicam inhibited both superoxide generation and lysosomal enzyme release (Kaplan et al, 1984).

When the stimulus was changed to PMA instead of FMLP, ibuprofen showed no significant inhibition of either superoxide generation or lysosomal release, whereas piroxicam continued to inhibit superoxide generation while having no effect on lysosomal release (Abramson, 1984).

In vivo studies of the NSAIDs on normal volunteers have substantiated the above in-vitro results (Abramson, 1985). Other in vivo studies have indicated that neutrophil adherence is inhibited by oral and IV administration of aspirin, indomethacin and phenylbutazone (MacGregor et al, 1974; MacGregor, 1976). In addition, recent studies have shown that the newer NSAIDs such as ibuprofen, fenoprofen and sulindac all have significant inhibition of neutrophil adherence, with sulindac having the least effect (Abramson et al, 1985).

Both the above in-vitro and in vivo studies have indicated that neutrophils have differing susceptibility, not only to each NSAID, but also to the applied stimulus. This may suggest that all neutrophil responses are not necessarily connected to a common pathway that is inhibited by the NSAIDs, but may involve several differing mechanisms.

iii) Gold Salts

The inhibition of neutrophils by gold compounds is suggested to be an important mechanism of action in their use for the treatment of rheumatoid arthritis. Auranofin, an orally effective chrysotherapeutic agent, produces a marked reduction in the extracellular level of β -glucuronidase and lysosyme released from zymosan-stimulated rat neutrophils (DiMartini et al, 1977). In addition, human neutrophils challenged with IgG-RF immune complexes showed marked inhibition of lysosomal enzyme release at μ g concentrations of auranofin (Finkelstein et al, 1977). Other studies using the same drug have shown significant inhibition of phagocytic cell release of superoxide (Roisman et al, 1982).

iv) Other Anti-Inflammatory agents

The reason for therapeutic properties of antimalarial drugs such as chloroquine and hydroxychloroquine in

inflammatory diseases is unclear, but may be related to their effects on neutrophils, in addition to effects on NK cells and monocytes (Ferrante & Goh, 1984). A recent study by Ferrante et al (1986) showed that several anti-malarial drugs inhibited neutrophil activity particularly the iodination reaction and locomotion.

The effects of other anti-inflammatory drugs such as penicillamine on neutrophil functions have not been well described, although Darth & Kahan (1984) reported that rat neutrophil chemotaxis to FMLP and C5a was blocked by cyclosporin, although no effect on neutrophil phagocytosis was observed.

CHAPTER TWO

UPTAKE OF ^3H -DEOXYGLUCOSE AS A MICROASSAY OF HUMAN
NEUTROPHIL AND MONOCYTE ACTIVATION

For clarity, materials and methods used for the experiments in this thesis are described in the relevant chapters respectively. In this chapter, the development of a new method to assay neutrophil activation is described.

Introduction

The main energy source for neutrophils and monocyte-macrophage series comes from the metabolism of glucose. The glycolytic pathway provides energy for adherence, chemotaxis, phagocytosis and other vital cell functions (Borregaard & Herlin, 1982), while the hexose monophosphate shunt provides energy for the generation of oxygen free-radicals which are vital for microbicidal activities and mediation of inflammatory tissue destruction (Baehner et al, 1970; Klebanoff, 1975; Babior, 1978).

The transport and uptake of glucose into phagocytic cells can be measured by radiometric techniques. For such assays analogues of glucose that are not metabolised should be used so that their accumulation in phagocytic cells provide a quantitative assessment of their state of activation. The 2-Deoxy-D[1-³H] glucose uptake assay has been used for the study of macrophage activation in guinea pigs and other animal species (Bonventre & Mukkada, 1974; Mukkada & Bonventre, 1975; Bonventre et al, 1977; McCormack et al, 1981). We now adapt this method to human neutrophils and monocytes and describe a simple, rapid and reproducible microassay technique suitable for use in experimental immunology.

Materials and Methods

Reagents

The 2-Deoxy-D[1-³H] glucose was purchased from Radiochemical Centre, Amersham, UK. It had a specific activity of 15Ci/mmol or 90.9 mCi/mg. The radiochemical concentration was 1.0 mCi/ml.

Sodium azide and formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Company, St Louis, Missouri, USA. Sodium fluoride and potassium cyanide were obtained from May & Baker, Sydney, Australia.

Isolation of neutrophils and monocytes

Heparinised blood from healthy volunteers was obtained by venepuncture. Five ml of blood was carefully layered onto 3ml of Mono-Poly Resolving Medium (Flow Laboratories, Virginia, USA) and centrifuged at 1,000G for 30 min. This resulted in separation of mononuclear cells at the top band and neutrophils at a second band (Ferrante & Thong, 1980). The neutrophils were removed, washed twice and resuspended in glucose-free Dulbecco's balanced salt solution (BSS). These neutrophils were of >97% purity (Ferrante & Thong, 1980).

The percoll gradients were prepared as previously described (Giddings et al, 1980). Briefly, one part of 10x phosphate buffered saline (PBS) was added to nine parts of percoll (Pharmacia, Sweden), and diluted with PBS to give a starting density of 1.0699 g/ml. Aliquots of 8ml of this solution were placed in plastic tubes in a Beckman ultracentrifuge and spun at 26,500g for 20 min. at room temperature. The mononuclear cells from the top band (see above paragraph) were washed and resuspended in Dulbecco's BSS, layered onto the percoll gradients and spun at 800G for 20 min. at room temperature. Monocytes of >90% purity were obtained from the topmost of the three bands.

³H-Deoxyglucose uptake microassay

In most experiments, 5×10^5 cells in 0.1 ml of Dulbecco's BSS were delivered to each well of a round bottom microtitre plate (Linbro/Titertek, Flow Laboratories, Virginia, USA). Another 0.1 ml of Dulbecco's BSS containing ³H-deoxyglucose was delivered to each well to give a final concentration of 0.78 uCi/ml. The microtitre plate was incubated at 37°C in a humidified air atmosphere for 30 min, then centrifuged at 4°C and 800g for 5 min. and 0.05 ml of supernatant was removed for determination of radioactivity in a LKB liquid scintillation counter. The absolute uptake of ³H-deoxyglucose was calculated from the following formula:

Deoxyglucose uptake

$$= \text{Total DPM added} - \text{DPM in supernatant}$$

The results were also calculated as percent deoxyglucose uptake as follows:

$$\% \text{ deoxyglucose uptake} = \frac{\text{Total DPM} - \text{DPM in supernatant}}{\text{Total DPM}}$$

Experiments were performed in triplicate and the results expressed as mean \pm SD.

In some experiments, varying concentrations of cells, deoxyglucose and different incubation time periods were used (see below).

In other experiments when FMLP or metabolic inhibition were employed, adjustments were made to the volumes of cells and deoxyglucose so that the final volume in each microtitre well was kept constant at 0.2 ml.

Statistical analysis

The student's test was used for statistical analysis of the data.

Results

Time-Course relationships

Preliminary experiments indicated that a cell number of 5×10^5 per microtitre well and a ^3H -deoxyglucose concentration of $0.78 \mu\text{Ci/ml}$ were suitable for the assay. Cells from a single donor were incubated for varying time periods using these parameters. The results (Figure 2.1) show that deoxyglucose uptake for both neutrophils and monocytes reach a plateau by 30 min. This time period was used in all subsequent experiments.

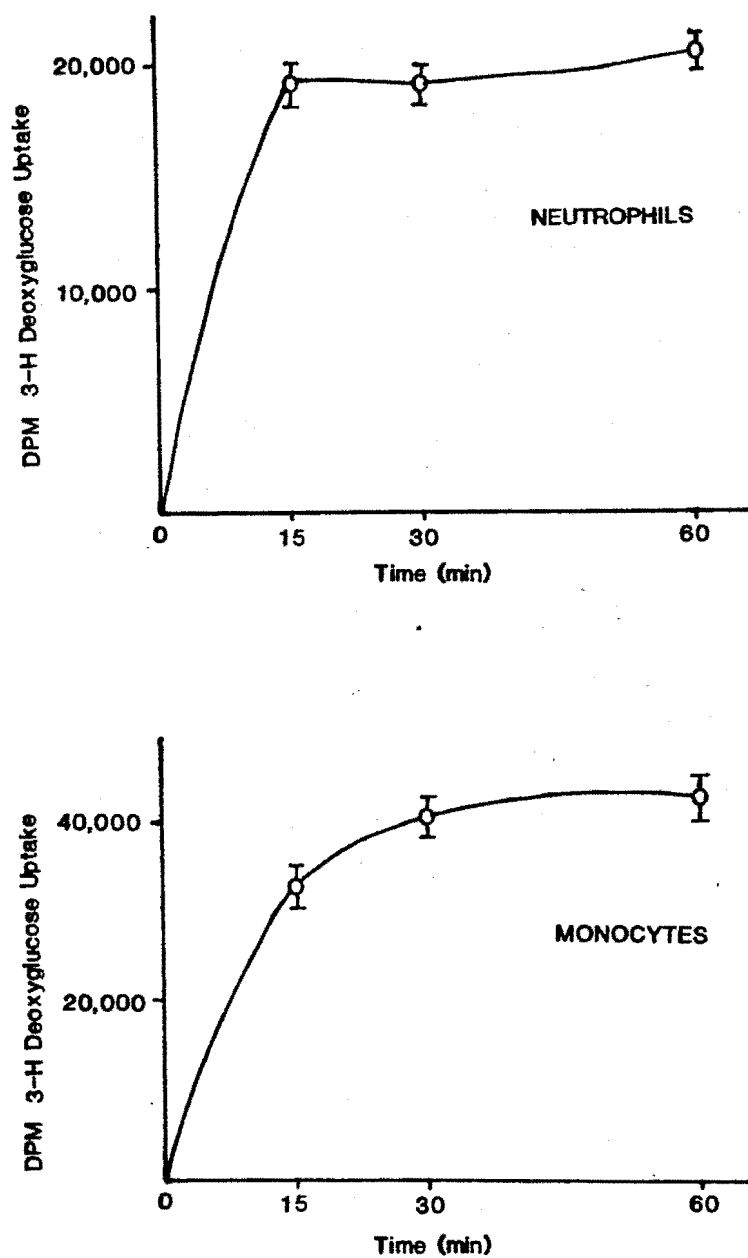


Figure 2.1 Time-course relationships for ^3H -deoxyglucose uptake by neutrophils and monocytes. The cell number was 5×10^5 and ^3H -deoxyglucose concentration $0.78 \mu\text{Ci/ml}$. The experiments were performed in triplicate and the results expressed as mean \pm S.D.

Dose-response relationships

In order to determine suitable concentrations of ^3H -deoxyglucose, cell numbers were standardised at 5×10^5 per microtitre well and incubation time at 30 min. The results (Table 2.1) show that in the case of neutrophils, maximum percent deoxyglucose uptake of 31.7 ± 1.0 occurred at a ^3H -deoxyglucose concentration of 0.78 $\mu\text{Ci/ml}$. Decreasing the concentration to 0.39 $\mu\text{Ci/ml}$ decreased percent uptake to only 16.4 ± 2.7 and increasing the concentration to 1.56 $\mu\text{Ci/ml}$ also decreased percent uptake to 23.6 ± 0.1 . In the case of monocytes, although concentrations of ^3H -deoxyglucose of 0.39 and 0.78 $\mu\text{Ci/ml}$ produced similar percent uptakes of 26.2 ± 0.2 and 25.9 ± 0.2 respectively, increasing the concentration to 1.56 $\mu\text{Ci/ml}$ decreased percent uptake. Hence the concentration of 0.78 $\mu\text{Ci/ml}$ was chosen for subsequent experiments with both neutrophils and monocytes.

Cell number relationships

In the development of a microassay technique, it is important to minimise the number of cells used without sacrificing sensitivity. For these experiments the concentration of ^3H -deoxyglucose was fixed at 0.78 $\mu\text{Ci/ml}$ and the incubation time at 30 min. Table 2.2 shows that although 10×10^5 cells per well resulted in very high percent uptake, 5×10^5 cells per well produced adequate uptake for our requirements, while the

Table 2.1 Effect of varying concentrations of ^3H -deoxyglucose on its uptake by neutrophils and monocytes.

^3H -deoxyglucose concentration ($\mu\text{Ci/ml}$)	^3H -deoxyglucose uptake (mean \pm S.D.)			
	Neutrophils		Monocytes	
	DPM	Percent	DPM	Percent
0.39	9,201 \pm 1,556	16.4 \pm 2.7	14,623 \pm 1,514	26.2 \pm 1.0
0.78	36,521 \pm 1,218	31.7 \pm 1.0	28,675 \pm 180	25.9 \pm 0.2
1.56	53,706 \pm 1,935	23.6 \pm 0.1	54,014 \pm 3,690	22.9 \pm 1.5

Incubation time was 30 min, and cell number 5×10^5 . Results represent mean \pm S.D. of triplicate samples and are calculated as both DPM and percent uptake.

Table 2.2 Effect of varying numbers of neutrophils and monocytes on ^3H -deoxyglucose uptake.

Cell Number	^3H -deoxyglucose uptake (mean \pm S.D.)			
	Neutrophils		Monocytes	
	DPM	Percent	DPM	Percent
1×10^5	8,779 \pm 274	7.5 \pm 0.2	4,079 \pm 116	4.9 \pm 0.1
5×10^5	28,002 \pm 961	24.0 \pm 0.8	12,905 \pm 458	15.7 \pm 0.6
10×10^5	37,782 \pm 1,041	32.4 \pm 0.9	18,887 \pm 420	23.0 \pm 0.5

Incubation time was 30 min and final concentration of ^3H -deoxyglucose was 0.78 $\mu\text{Ci/ml}$ (0.16 μCi in 0.2 ml). Experiments were performed in triplicate and expressed as mean \pm S.D. in DPM or percent uptake.

use of 1×10^5 cells resulted in minimal uptake.

Effect of metabolic inhibitors

In order to determine the validity of this assay, we studied the effects of potassium cyanide and sodium azide, known inhibitors of oxidative metabolism (Borreagaard & Herlin, 1982; Kelly & Thong, 1984) and sodium fluoride, a known inhibitor of the glycolytic pathway (Thong & Currell, 1983), on the ^3H -deoxyglucose uptake of these phagocytic cells. The results (Table 2.3) showed significant inhibition of deoxyglucose uptake by all three metabolic poisons. In the case of neutrophils, sodium fluoride had a more pronounced effect than potassium cyanide or sodium azide. This is not an unexpected finding as the neutrophils lack mitochondria and rely mainly on the glycolytic pathway for energy generation.

Effect of FMLP and PMA

The chemotactic tripeptide, FMLP, and the phorbol ester, PMA are potent stimulators of phagocytic cells (Schiffmann et al, 1975; Becker, 1975; O'Flaherty et al, 1979). The binding of FMLP or PMA to specific receptors of neutrophils and monocytes results in enhancement of adherence, chemotaxis, respiratory burst and degranulation. The results (Table 2.4) show that FMLP at biologically active concentrations of 10^{-5}M to 10^{-7}M

Table 2.3 Effect of metabolic inhibitors on ^3H -deoxyglucose uptake by neutrophils and monocytes.

Metabolic Inhibitors	^3H -deoxyglucose uptake (mean \pm S.D.)			
	Neutrophils		Monocytes	
	DPM	Percent	DPM	Percent
Control	39,095 \pm 1,563	34.7 \pm 1.3	23,786 \pm 1,035	21.6 \pm 1.0
Potassium cyanide (10mM)	21,211 \pm 578	18.8 \pm 0.5**	12,730 \pm 245	11.6 \pm 0.2**
Sodium azide (10mM)	33,312 \pm 611	29.6 \pm 0.5*	15,684 \pm 427	14.3 \pm 0.4**
Sodium fluoride (50mM)	10,323 \pm 172	9.1 \pm 0.1**	11,621 \pm 188	10.5 \pm 0.1**

Incubation time was 30 min, cell number 5×10^5 and ^3H -deoxyglucose concentration 0.78 $\mu\text{Ci/ml}$ in a final volume of 0.2 ml. Results are expressed as mean \pm S.D. of triplicate samples in either DPM or percent uptake.

* $p < 0.01$

** $p < 0.001$

Table 2.4 Effect of varying concentrations of FMLP on ^3H -deoxyglucose uptake by neutrophils and monocytes.

FMLP concentration	^3H -deoxyglucose uptake (mean \pm S.D.)			
	Neutrophils		Monocytes	
	DPM	Percent	DPM	Percent
0	16,839 \pm 717	14.7 \pm 0.6	14,248 \pm 523	11.6 \pm 0.4
10^{-7}M	22,983 \pm 1678	20.1 \pm 1.4*	22,428 \pm 305	18.3 \pm 0.2**
10^{-6}M	29,567 \pm 781	25.8 \pm 0.7**	20,534 \pm 318	16.7 \pm 0.3**
10^{-5}M	29,220 \pm 661	25.6 \pm 0.6**	21,662 \pm 542	17.7 \pm 0.4**

Incubation time was 30 min, cell number 5×10^5 and ^3H -deoxyglucose concentration 0.78 $\mu\text{Ci/ml}$ in a final volume of 0.2 ml. Experiments were performed in triplicate and expressed as mean \pm S.D. in both DPM and percent uptake.

* $p < 0.01$

** $p < 0.001$

stimulated significant increases in ^3H -deoxyglucose uptake by neutrophils and monocytes compared to untreated cells. The optimal concentration for PMA appears to be 0.1 $\mu\text{g/ml}$; higher concentrations resulted in decreased uptake because of toxicity (Table 2.5).

Discussion

In 1933, Balridge and Gerard (1933) first observed that phagocytic cells display a burst of respiratory activity when presented with a phagocytic stimulus. Some 25 years later, Sbarra and Karnovsky (1959) discovered that the increased oxygen uptake was the result of glucose metabolism via the glycolytic pathway and the hexose monophosphate shunt. Current understanding suggests that the energy required for movement and phagocytosis is provided by glycolysis (Borreagaard & Herlin, 1982) while the hexose monophosphate shunt is involved in the production of toxic oxygen free-radicals used for microbial killing (Babior, 1978).

Increased glucose transport following activation of mouse and guinea pig peritoneal macrophages by immunologic and phagocytic stimuli has been reported by a number of investigators (Nathan et al, 1971; Bonventre & Mukkada, 1974). Bonventre and co-workers (1977) showed that the 2-deoxy analogue of glucose is particularly useful in this regard because although it shares a

Table 2.5 Effect of varying concentrations of PMA on ^3H -deoxyglucose uptake by neutrophils and monocytes

PMA concentration ($\mu\text{g/ml}$)	^3H -deoxyglucose uptake (mean \pm S.D.)			
	Neutrophils		Monocytes	
	DPM	Percent	DPM	Percent
0	33,724 \pm 1,017	33.6 \pm 1.0	10,541 \pm 600	10.3 \pm 0.6
0.01	32,738 \pm 1,955	32.6 \pm 1.9	16,131 \pm 1,270	15.8 \pm 1.2*
0.1	46,470 \pm 1,284	46.2 \pm 1.3**	17,975 \pm 1,323	17.6 \pm 1.3*
1.0	5,826 \pm 557	5.8 \pm 0.6**	12,055 \pm 1,105	11.8 \pm 1.1

Incubation time was 30 min, cell number 5×10^5 and ^3H -deoxyglucose concentration 0.78 $\mu\text{Ci/ml}$ in a final volume of 0.2 ml. Experiments were performed in triplicate and expressed as mean \pm S.D. in both DPM and percent uptake.

* $p < 0.01$

** $p < 0.001$

common membrane transport system with glucose, it is not metabolised after initial phosphorylation. McCormack et al, (1981) highlighted the sensitivity, reproducibility and other advantages of the ^3H -deoxyglucose uptake assay over the standard indirect macrophage migration inhibitory assay for the detection of lymphokine-induced macrophage activation. We now demonstrate the applicability of this assay to human monocytes, an immature precursor of tissue macrophages, and to human neutrophils, a phagocytic cell of separate lineage to monocytes-macrophages.

Instead of using glass coverslips for phagocytic cells to adhere to as in previous methods, we used microtitre trays. This offers several advantages. Firstly, precise numbers of cells and quantities of reagents can be dispensed into each microtitre well, and the uptake of ^3H -deoxyglucose can be related directly to cell numbers thus obviating the need for protein estimations. Secondly, the requirement for small numbers of cells means that the assay can be performed with less than 3 ml of blood, and therefore is applicable to diagnostic use in paediatric practice. Thirdly, there is excellent intra-experiment reproducibility as shown by the small standard deviations of triplicate samples. The microassay has been standardised to a ^3H -deoxyglucose concentration of 0.78 $\mu\text{Ci/ml}$

and cell number of 5×10^5 in a final volume of 0.2 ml per microtitre well; and incubation time of 30 min. The validity of the microassay is demonstrated by its susceptibility to inhibition by metabolic poisons such as potassium cyanide, sodium azide and sodium fluoride, and stimulation by the chemotactic tripeptide, FMLP, and the phorbol ester, PMA.

This microassay may be suitable for use for experimental laboratory investigations and clinical assessment of phagocytic cell activity in vitro.

CHAPTER THREE

MODULATORY EFFECTS OF STREPTOCOCCUS MUTANS ON HUMAN NEUTROPHIL ADHERENCE AND DEOXYGLUCOSE UPTAKE

Introduction

Streptococcus mutans is the major bacterial species associated with dental caries in man (Krasse et al, 1968; Loesche et al, 1975; Hamada & Slade, 1980). Extensive epidemiological studies indicate that serotype c is the most prevalent of the seven serotypes (a-g) in dental plaque (Duany et al, 1972; Bratthall, 1970; 1972; Grenier et al, 1973; Perch et al, 1974; Shklair & Keene, 1974; Hamada et al, 1976; Bright et al, 1977; Qureshi et al, 1977; Masuda et al, 1979). Implantation experiments show also that serotype c has a much greater propensity than serotype a for colonising the oral cavity (Svanberg & Loesche, 1978). Taken together, these observations suggest that *Strep. mutans* serotype c has biological advantages over other serotypes, but to our knowledge, no studies have yet addressed this question.

It is well recognised that successful pathogens have the ability to evade host immunological responses (Densen & Mandell, 1980; Spitznagel, 1983), since polymorphonuclear neutrophils (PMNs) represent between 95-97% of the immunocytes in crevicular fluid (Attstrom & Egelberg, 1970; Scully & Challacombe, 1979) and the first line of defence against extracellular microbes (Johnston, 1982). The present study was carried out to examine the direct interaction between *Strep. mutans* and neutrophils. In particular, we sought to determine whether *Strep. mutans* serotype c is more able than other less predominant serotypes to suppress neutrophil activation as measured by adherence and deoxyglucose uptake. We chose these assays because adherence is the earliest observable change in neutrophil behaviour following activation, and perhaps one of the most crucial (Palmlad et al, 1981; Gallin, 1985; Seow & Thong, 1986a). Glucose uptake provides energy for neutrophils to adhere (Borregaard & Herlin, 1982; Kelly & Thong, 1984), as well as generate oxygen free-radicals (Johnston, 1982; Klebanoff, 1980).

Materials and Methods

Strep. mutans serotypes

The five strains (four serotypes) of *Strep. mutans* used in these experiments were obtained from Dr A. H.

Rogers, University of Adelaide (Rogers, 1976). They were designated (serotype in parenthesis): B2(c), 13M(c), E49(a), B13(d) and OMZ(d,g). They were grown anaerobically in mycoplasma broth (Gibco Diagnostics, Madison), supplemented with 0.2% glucose and hemin. In some experiments, 10% sucrose was substituted for glucose. The purity of the cultures was checked in each case by noting colony morphology on blood agar plates and by Gram staining. The bacteria were harvested by centrifugation at 10,000g for 15 min. at 4°C, washed twice in normal saline and resuspended in medium 199. Cell concentrations were determined by diluting stock bacterial suspensions and counting in a Neubauer haemocytometer.

Treatment of Strep. mutans B2(c) with physical and chemical agents.

Heat. A portion of a strain of Strep. mutans B2(c) at a concentration of approximately 40×10^6 /ml were incubated at 75°C for one hour in a water bath. Heat-treated organisms were cooled at 37°C before use.

Formalin. Strep. mutans B2(c) at concentrations of approximately 40×10^6 /ml were suspended in medium 199 containing 2% formalin. The mixture was incubated for 24 hours at 37°C, washed twice and resuspended to its original concentration with medium 199.

Ultrasonication. A Branson sonicator with probe tip set at 6 was used to sonicate a *Strep. mutans* B2(c) suspension which had been adjusted to a concentration of approximately 40×10^6 /ml. Sonication was achieved by applying intermittent bursts of 15 secs. for 3 hours in an ice bath. Microscopy of the sonicated preparations revealed 80% lysis of bacteria. The sonicated preparations were centrifuged at 2000g for 10 mins. which sedimented any remaining whole cells. The resulting supernatant fluid was used in subsequent studies.

Isolation of Neutrophils

Heparinised blood of healthy volunteers was obtained by venepuncture. Neutrophils were obtained by a one-step centrifugation procedure using Mono-Poly Resolving Medium (Flow Laboratories, Virginia. USA) as previously described (Ferrante & Thong, 1980). The neutrophils were harvested from the second band, washed twice and resuspended in medium 199 to a concentration of approximately $4-5 \times 10^6$ /ml. The cells were of >97% purity.

Neutrophil adherence microassay

The neutrophil adherence assay which employs the use of nylon fibre microcolumns has been previously described (Thong & Currell, 1983; Li et al, 1985) and

shown in Figure 3.1. Briefly, the nylon fibre microcolumns were prepared by carefully weighing out 10mg lots of teased nylon fibre (Olympic Products, Queensland, Aust.). Each lot was placed into a 100 μ l disposable pipette tip (Stockwell Scientific, California) so as to occupy the centre 2cm portion of the pipette tip. An aliquot of 100 μ l of each neutrophil suspension, with or without bacteria, was pipetted into each nylon fibre microcolumn. After an incubation time of 5 min. at 37°C and high humidity in order to allow for contact between neutrophils and nylon fibre, the microcolumns were placed in a specially designed apparatus for extraction of the fluid by a vacuum suction pressure of 250 mbar, applied for 1 min., into disposable test tubes. The concentration of neutrophils was determined by the Neubauer haemocytometer and results calculated as follows:

$$\% \text{ adherence} = \frac{\text{Neutrophil conc. in effluent}}{\text{Neutrophil conc. in original neutrophil suspension}} \times 100$$

In some experiments, the results were further expressed as percent control and calculated as follows:

$$\% \text{ of control} = \frac{\% \text{ adherence of test (with bacteria)}}{\% \text{ of control (without bacteria)}} \times 100$$

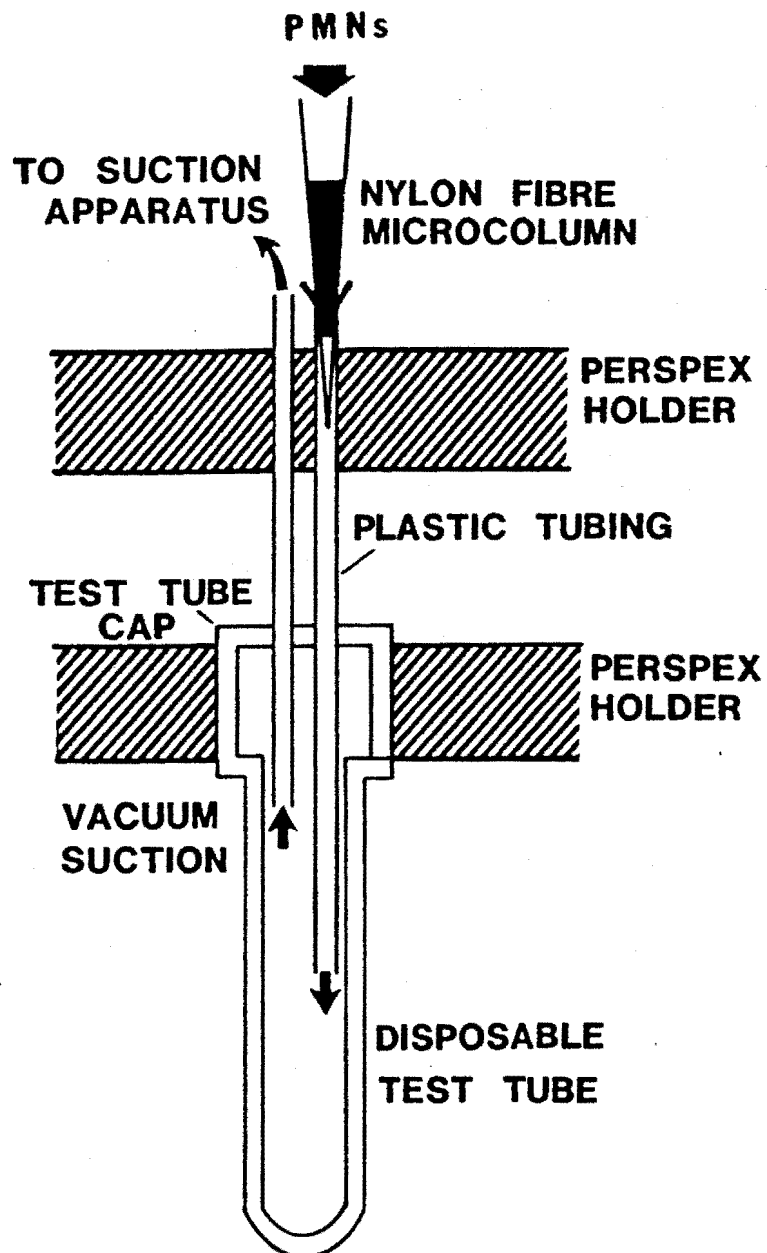


Figure 3.1 Diagrammatic representation of the microcolumn technique for PMN adherence.

Each experiment was performed in triplicate and the results expressed as mean \pm SE.

Deoxyglucose uptake

We used a newly developed deoxyglucose uptake microassay for these experiments (Seow et al, 1987e). This assay was modified from previously described techniques (McCormack et al, 1981). Approximately 5×10^5 neutrophils in 0.05ml of Dulbecco's BSS were delivered to each well of a round-bottom microtitre plate. Another 0.05ml of Dulbecco's BSS containing 50×10^5 bacteria was added to test wells. In addition, the tumour promotor, phorbol myristate acetate (PMA, Sigma Chemical Co. St Louis), in final concentrations of 2.5ug/ml or 0.1 ug/ml, was added to the wells containing the bacteria. Control wells received Dulbecco's BSS without bacteria and PMA. Another set of control wells received PMA without bacteria. A further 0.5 ml of Dulbecco's BSS containing 2-D³H-deoxyglucose (Radiochemical Centre, Amersham, UK) was added to each well to give a final concentration of 0.79 uCi/ml. The final volume in all wells was kept constant at 0.2ml. The microtitre plate was incubated at 37°C in a humidified-air atmosphere for 30 mins., centrifuged at 4°C and 800g for 5 mins., and 0.05ml of supernatant removed for determination of radioactivity in a LKB liquid scintillation counter. The uptake of ³H-deoxyglucose was calculated from this formula:

Deoxyglucose uptake

$$= \text{Total DPM added} - \text{DPM in supernatant}$$

$$\text{Percent Deoxyglucose uptake} = \frac{\text{Deoxyglucose uptake}}{\text{Total DPM added}}$$

Experiments were performed in triplicate and the results expressed as mean \pm S.D.

Statistical Analysis

The student's *t* test was used for statistical analysis of the results.

Results

Modulation of neutrophil adherence by Strep. mutans

Different strains of Strep. mutans were incubated with neutrophils at 37°C (Bacteria:neutrophil ratio of 10:1), before being placed into the nylon fibre micro-columns for assay of adherence.

The results of seven experiments using neutrophils from 7 donors are summarised in Table 3.1. Both serotype c strains significantly suppressed neutrophil adherence to $74.8 \pm 3.2\%$ of control ($p < 0.001$), and $70.3 \pm 4.4\%$ of control ($p < 0.001$), [serotypes B2(c) and 13M(c), respectively]. In contrast, the other serotypes

TABLE 3.1 Direct effects of various strains of Streptococcus mutans on PMN adherence.

<u>Streptococcus mutans</u> strain (serotype)	PMN adherence % of control (mean \pm S.E.)	P value
B2(c)	74.8 \pm 3.2	< 0.001
13M(c)	70.3 \pm 4.4	< 0.001
E49(a)	96.5 \pm 4.0	N.S.
B13(d)	118.9 \pm 6.3	< 0.02
OMZ(d,g)	106.2 \pm 2.9	N.S.

The results are expressed as percent of control (see Methods section), and represent the mean \pm S.E. of 7 experiments using PMNs from 7 separate donors. The bacteria:PMN ratio was 10:1, and incubation time 5 min.

either had no noticeable effect on neutrophil adherence [serotypes E49(a), OMZ(d,g)], or actually enhanced neutrophil adherence [serotype B13(d)].

Effect of varying bacteria-neutrophil contact times on neutrophil adherence

To examine the effect of varying incubation times with Strep. mutans on neutrophil adherence, Strep. mutans E49(a), which has minimal effect on neutrophil adherence, and Strep. mutans B2(c), which decreased neutrophil adherence, were selected for study. The results of these experiments (Table 3.2) showed that the interaction time between the streptococci and neutrophils was very rapid. Within 2 min, Strep. mutans B2(c) depressed neutrophil adherence to 84.5 ± 2.2 percent of control ($p < 0.02$). This depression continued to be observed at 5 and 15 min. In contrast, Strep. mutans E49(a) showed insignificant effects on neutrophil adherence compared to control at all the bacteria-neutrophil contact times.

Effect of varying streptococci:neutrophil ratios on neutrophil adherence

Table 3.3 shows the effects of varying concentrations of Strep. mutans E49(a) and Strep. mutans B2(c) on neutrophil adherence. At the ratio of 0.1 bacteria:1 neutrophil, neutrophil adherence was not significantly

TABLE 3.2 Effects of varying incubation times with S. mutans B2(c) and S. mutans E49(a) on PMN adherence.

Incubation time (mins)	PMN adherence % of control (mean \pm S.E.)	
	<u>S. mutans</u> B2(c)	<u>S. mutans</u> E49(a)
2	84.5 \pm 2.2*	105.2 \pm 4.7
5	80.1 \pm 2.7*	106.0 \pm 4.5
15	82.6 \pm 2.9**	109.6 \pm 4.2

PMNs were incubated with bacteria using bacteria:PMN ratios of 10:1 for varying time periods at 37°C prior to PMN adherence assay. The experiments were done in triplicate and the results expressed as percent of control (mean \pm S.E.)

* p < 0.02

** p < 0.05

TABLE 3.3 Effects of varying concentrations of S. mutans B2(c) and S. mutans E49(a) on PMN adherence.

Bacteria:PMN ratio	PMN adherence % of control (mean \pm S.E.)	
	<u>S. mutans</u> B2(c)	<u>S. mutans</u> E49(a)
100:1	68.6 \pm 2.6*	103.6 \pm 2.4
10:1	84.3 \pm 2.6**	96.4 \pm 6.7
1:1	89.6 \pm 5.7	103.6 \pm 0.9
0.1:1	93.1 \pm 4.1	99.2 \pm 3.6

PMNs were incubated with bacteria at varying bacteria:PMN ratios for 5 min at 37°C prior to PMN adherence assay. The experiments were performed in triplicate and the results expressed as mean \pm S.E. (percent of control).

* p < 0.001

** p < 0.01

different from controls for both strains of *Strep.* mutans. At the higher bacteria:neutrophil ratio of 10:1, *Strep.* mutans B2(c) decreased neutrophil adherence to 84.3 ± 2.6 percent of control. This was statistically significant ($p < 0.01$). At the bacteria:neutrophil ratio of 100:1, the effects were even more marked at 68.6 ± 2.6 percent of control ($p < 0.001$). In contrast, in the case of *Strep.* mutans E49(a), no significant effects on neutrophil adherence were observed at any of the bacteria:neutrophil ratios tested.

Effect of *Strep.* mutans cultured in 10% sucrose on neutrophil adherence

Growth of *Strep.* mutans in the presence of sucrose is associated with increased virulence (Michalek et al, 1977). Therefore, experiments were conducted using strains B2(c) and E49(a) grown in mycoplasma broth with 10% sucrose to examine whether changes in neutrophil interactions could be observed compared to *Strep.* mutans grown in standard media containing 0.2% glucose. The results of these experiments are shown in Table 3.4. In the case of strain B2(c), organisms grown in the standard media depressed neutrophil adherence to 59.5 ± 3.9 percent of control. Organisms grown in media containing 10% sucrose showed a comparable depression of neutrophil adherence of 63.4 ± 3.3 percent of control, showing that growth in sucrose media does not influence

TABLE 3.4 Direct effects of S. mutans cultured in 10% sucrose on PMN adherence.

<u>S. mutans</u>	PMN adherence % of Control (mean \pm S.E.)	
	Std media (0.2% Glucose)	10% Sucrose
B2(c)	59.5 \pm 3.9*	63.4 \pm 3.3*
E49(a)	101.3 \pm 1.9	104.0 \pm 5.3

PMNs were incubated with bacteria at the bacteria:PMN ratio of 10:1 for 5 min at 37°C prior to PMN adherence assay. The experiments were performed in triplicate and the results expressed as percent of control (mean \pm S.E.).

* $p < 0.01$ compared to control, $p > 0.1$ compared with each other.

the interaction of neutrophils with Strep. mutans B2(c). Similarly, for strain E49(a), there were no significant changes in neutrophil adherence compared to control for organisms grown in standard media as well as for those grown in sucrose.

Effect of physical and chemical treatments of Strep. mutans on neutrophil adherence

Since it is most likely that the modulatory effects of Strep. mutans reside in the bacterial cell wall, experiments were performed to determine if disruption of the bacterial cell wall affects neutrophil adherence.

Figure 3.2 shows the effects of heat, formalin and sonication on Strep. mutans B2(c). Untreated bacteria depressed percent neutrophil adherence to $40.5 \pm 3.9\%$ compared to a control value of $60.2 \pm 3.9\%$. The difference is statistically significant ($p < 0.02$). However, after treatment with heat, formalin and sonication, respectively, neutrophil adherence was similar to control values, showing that an integral bacterial cell wall is necessary for optimal interaction with neutrophils.

Effect of Strep. mutans on neutrophil deoxyglucose uptake

Energy for adherence and generation of oxygen free-

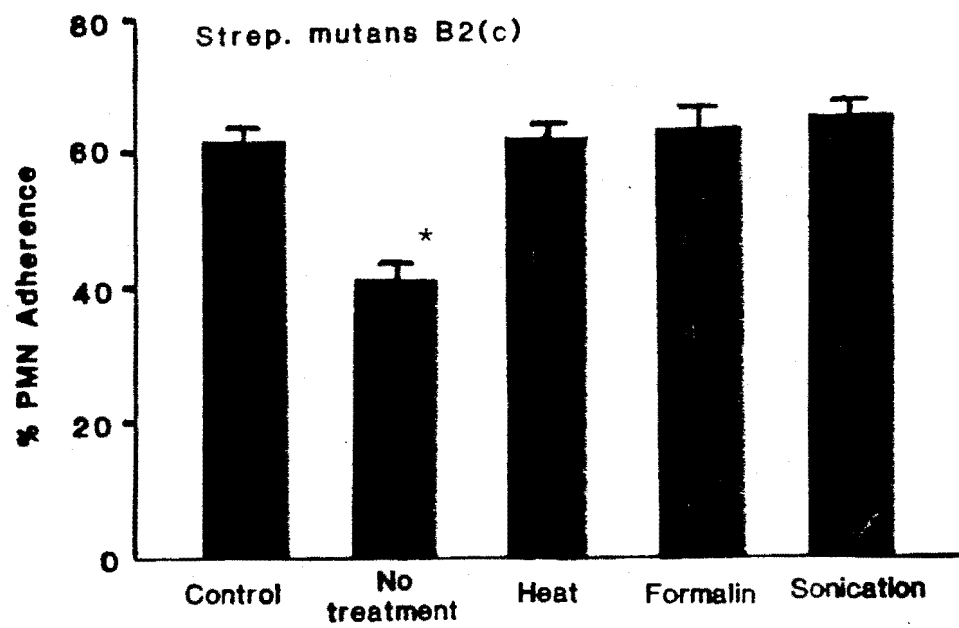


Figure 3.2 Effects of various physical and chemical treatments of *S. mutans* B2(c) on PMN adherence

* $p < 0.02$

radicals in phagocytic cells is provided by glucose metabolism via the glycolytic pathway and hexose-monophosphate shunt (Borregaard & Herlin, 1982; Klebanoff, 1980). One way in which bacteria can inhibit neutrophil adherence may be by interfering with energy requirements. This possibility can be investigated by measuring the cellular accumulation of ^3H -deoxyglucose, an analogue of glucose which can be taken up but not metabolised (Seow et al, 1987e; McCormack et al, 1981).

Both *Strep.* mutans strains B2(c) and E49(a) on their own did not affect the deoxyglucose uptake of neutrophils appreciably compared to controls ($p > 0.1$, data not presented). However, the addition of PMA, a potent neutrophil stimulant (Thong & Currell, 1983; Repine et al, 1974; Kelly et al, 1985), caused marked effects of these bacteria on neutrophil deoxyglucose uptake (Figure 3.3). In the presence of PMA at a suboptimal concentration of $0.1 \mu\text{g/ml}$, percent deoxyglucose uptake by neutrophils alone was not stimulated compared to a control value of 23.1 ± 0.2 ($p > 0.1$). However, in the presence of this substimulatory concentration of PMA, *Strep.* mutans B2(c) depressed percent neutrophil deoxyglucose uptake to only 18.8 ± 0.2 ($p < 0.001$). In contrast, *Strep.* mutans E49(a) increased percent neutrophil deoxyglucose uptake to 26.5 ± 0.6 ($p < 0.01$, compared to control).

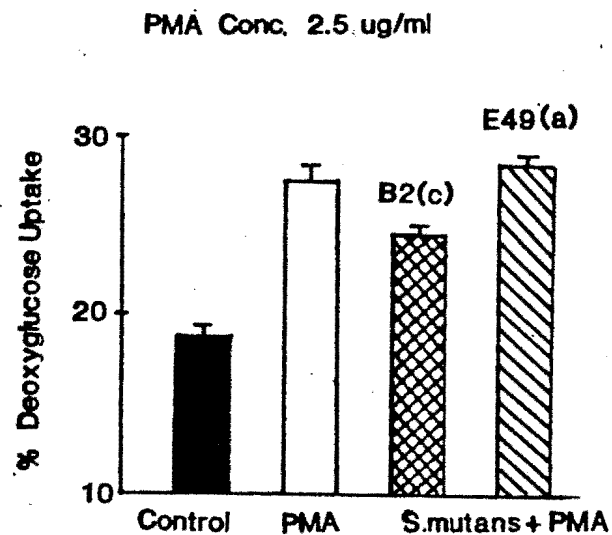
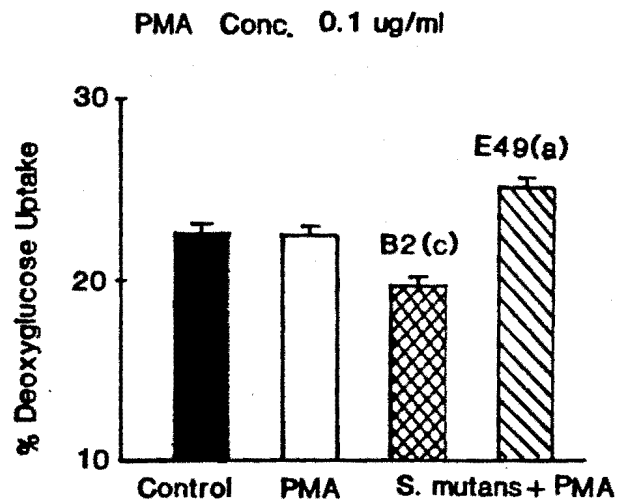


Figure 3.3 Effect of *S. mutans* B2(c) and E49(a) on PMA-augmented PMN deoxyglucose uptake. There was significant suppression of deoxyglucose uptake in B2(c) treated PMNs at suboptimal ($p < 0.001$) and optimal ($p < 0.001$) concentrations of PMA.

Similar effects were observed at the higher PMA concentration of 2.5 ug/ml. At this concentration, PMA stimulated percent deoxyglucose uptake to 27.7 ± 1.7 , compared to a control value of 18.8 ± 0.5 ($p < 0.001$). However, the addition of Strep. mutans B2(c) depressed this stimulation to 24.5 ± 0.3 ($p < 0.05$). In contrast, the addition of Strep. mutans E49(a) did not change percent neutrophil deoxyglucose uptake appreciably compared to that seen at this concentration of PMA alone (28.2 ± 0.4 vs 27.7 ± 1.7 , $p > 0.1$).

The above results indicate that Strep. mutans B2(c) is able to suppress PMA-augmented deoxyglucose uptake in neutrophils.

Discussion

The results of the present study show that both strains of Strep. mutans serotype c have the capacity to suppress neutrophil adherence, while the three strains of other serotypes did not. This inhibitory effect on neutrophil adherence was observed as early as 2 mins. after contact between neutrophil and bacteria. It was best demonstrated at bacteria:neutrophil ratios of 10:1 and 100:1, but not 1:1. Culture of bacteria in high concentrations of sucrose did not alter their neutrophil adherence modulatory properties, but treatment of the bacteria with heat, formalin and ultrasonication

resulted in loss of this capacity. Augmentation of neutrophil deoxyglucose uptake by the tumour promotor, phorbol myristate acetate, was also suppressed by *Strep. mutans* serotype c.

These results indicate that serotype c of *Strep. mutans*, the predominant serotype found in the oral cavity, and the serotype with the greatest propensity for colonisation (Duany, 1972; Masuda et al, 1979) has an unique capacity to suppress neutrophil activation not shown by other serotypes. These findings may have biological relevance, as successful bacterial colonisation of the oral cavity would depend in part on the ability of bacteria to reduce or avoid activation of neutrophils, and the resultant release of lysosomal contents and oxygen free radicals with microbicidal properties (Densen & Mandell, 1980; Spitznagel, 1983). In support of this conclusion are previous studies which show that neutrophils comprise the majority of the immunocytes in crevicular fluid, and constitute one of the first lines of defence against microbial invasion in the oral cavity (Attstrom & Egelberg, 1970; Scully & Challacombe, 1979). Since antibody directed against *Strep. mutans* is practically non-existent following natural infection with this bacteria (Hamada & Slade, 1980; McGhee & Michalek, 1981), neutrophil mediated non-specific host defence mechanisms assume greater promi-

nence. Thus the particular ability of *Strep. mutans* serotype c to avoid activation of neutrophils may confer on it an advantage over other serotypes in its establishment in the oral cavity.

The capacity to evade destruction by phagocytic cells is a general phenomenon seen in a wide range of pathogenic microbes (Densen & Mandell, 1980; Spitznagel, 1983). Several reports are available on the evasion mechanisms of streptococcal species other than *Strep. mutans*. These include the anti-phagocytic M protein (Foley & Wood, 1959; Gemmell et al, 1981), and the leukocidins of group A *Streptococci* (Hirsch et al, 1963; Ofek et al, 1970; Sullivan & Mandell, 1980). Our data indicate that the capacity of *Strep. mutans* serotype c to inhibit neutrophil adherence may depend on the integrity of the bacterial cell wall, as alteration by physical and chemical agents abrogated this effect. This data is consistent with previous studies on other bacterial species which show that some anti-phagocytic mechanisms reside in bacterial cell walls (Smith, 1977; Orskov, 1978). The structural components of the cell wall of *Strep. mutans* include serotype carbohydrate, teichoic acid and protein interspersed throughout the peptidoglycan layer (McGhee & Michalek, 1981). Minor variations between these components among the serotypes may account for their differences in interactions with

neutrophils, but a clearer understanding must await molecular studies.

CHAPTER FOUR

MODULATION OF POLYMORPHONUCLEAR NEUTROPHIL ADHERENCE BY PULPOTOMY MEDICAMENTS: EFFECTS OF FORMOCRESOL, GLUTARAL- DEHYDE, EUGENOL AND CALCIUM HYDROXIDE

Introduction

The pulpotomy technique is now an accepted procedure for treating vital primary teeth with carious pulp exposures. The rationale of this procedure is that removal of coronal infected and inflamed pulpal tissue may allow retention of the tooth with vital root tissues. Medicaments commonly used following a pulpotomy procedure include formocresol, zinc oxide-eugenol, and calcium hydroxide (Frankl, 1972). More recently, glutaraldehyde has been suggested as a better alternative to formocresol due to its lower tissue toxicity (Lekka et al, 1984). Pulpotomy medicaments are used to kill bacteria remaining in the pulp and to preserve vital root pulp. Although clinical studies on

pulpotomy have reported high success rates (Rolling & Thylstrup, 1975; Doyle et al, 1962; Magnusson, 1978; Law & Lewis, 1964), histological studies have given disappointing results (Magnusson, 1980; Rolling et al, 1976), notably chronic pulpal inflammation, necrosis and internal resorption. Most authors have attributed poor histological sequelae to the lack of local tissue compatibility with commonly used pulpotomy medications (Loos et al, 1973). In addition, systemic effects have caused concern among clinicians (Myers et al, 1981; Myers et al, 1978; Lewis & Chester, 1981).

Polymorphonuclear neutrophils (PMNs) are phagocytic cells with important roles in host defence (Johnston, 1982), but inappropriate and uncontrolled stimulation of these cells can lead to their accumulation in excessive numbers resulting in tissue damage (Weissman et al, 1980). Whether or not pulpotomy medicaments have the capacity to activate neutrophils has not been studied. It is postulated that stimulation of neutrophils by pulpotomy medicaments may contribute to the chronic inflammatory changes seen with their use. In this study, the effects of some of these medicaments on neutrophil adherence, the earliest observable change in neutrophil behaviour following activation, are examined. Adherence of neutrophils to vascular endothelium is a

prerequisite for subsequent diapedesis and chemotaxis into the perivascular compartment, and hence of paramount importance in the initiation of the inflammatory response (McGillen et al, 1980; Anderson et al, 1984).

Materials and Methods

Pulpotomy medicaments selected for this study included formocresol, glutaraldehyde, eugenol, and calcium hydroxide, all commonly used in paediatric dentistry.

Formocresol (Creighton Pharmaceuticals, Sydney, Australia) in the form of Buckley's formula (19% formaldehyde, 35% cresol), was dissolved in absolute ethanol at a concentration of 1:5 (vol/vol), and further dilutions made in medium 199. Appropriate control solutions also were prepared with ethanol in medium 199.

Glutaraldehyde (Sigma Chemical Co., St. Louis, USA) was prepared in the recommended endodontic concentration of 2% aqueous solution, and subsequent dilutions were made in medium 199.

Eugenol BP (David Craig Chemicals, Brisbane, Australia) was dissolved in absolute ethanol at a

concentration of 1:5 (vol/vol). Subsequent dilutions were made using medium 199.

Calcium hydroxide (Otto & Co., Frankfurt, Germany) was prepared in a stock suspension of 10mg/ml made up in medium 199, and further dilutions made in the same medium. A fine suspension was obtained by vigorous shaking.

Neutrophil adherence

Neutrophils were purified from heparinised blood of healthy donors by one-step centrifugation procedures on a resolving medium (Flow Laboratories, Virginia, USA) as previously described (Ferrante & Thong, 1980). The neutrophils were harvested from the second band, washed twice, and resuspended in medium 199. They were of >97% purity.

The neutrophil adherence assay was performed using nylon fibre microcolumns as previously described (Thong & Currell, 1983). Briefly, the nylon fibre microcolumns were prepared by carefully weighing out 10mg lots of teased nylon fibre. These were placed in 100 ul disposable pipette tips so as to occupy the centre 2cm portion of the 5cm pipette tip. Neutrophil suspensions with or without pulpotomy agents were adjusted to concentrations between $4-6 \times 10^6$ cells/ml and 100ul

was delivered into each nylon microcolumn. After incubation for 5 min. at 37°C and high humidity in order to allow for contact between neutrophils and nylon fibre, the microcolumns were placed in a specially designed apparatus (Thong & Currell, 1983). The fluid was extracted by a vacuum suction pressure of 250 millibars, applied for 2 min. into disposable test tubes. The concentration of neutrophils was determined in a haemocytometer and the results calculated as follows:

$$\% \text{ adherence} = 100 - \frac{\text{Neutrophil conc.in effluent}}{\text{Neutrophil conc.in original suspension}} \times 100$$

Results were expressed as mean \pm SD of triplicate samples. In some experiments, the results were expressed further as a percentage of control and calculated as follows:

$$\% \text{ of control} = \frac{\% \text{ adherence of test sample}}{\% \text{ adherence of control sample}} \times 100$$

The student's t-test was used for statistical analysis of the results.

Viability studies

The viability of neutrophils was determined by the

trypan blue dye exclusion test (McLimmans et al, 1957). Briefly, the neutrophil suspensions were incubated with 2% trypan blue for 5 min. and the percentage of stained cells assessed by microscopy.

Results

Effects of varying concentrations of medicaments on neutrophil adherence

The results show that with the exception of glutaraldehyde, incubation with high concentrations of pulpotomy medicaments causes lysis of neutrophils. With lower concentrations, adherence of neutrophils was affected markedly (Table 4.1).

Formocresol at an intermediate concentration of 1:10,000 caused neutrophil adherence to be decreased to 41.5 ± 7.5 percent of controls ($p < 0.01$). In contrast, at a much lower concentration of 1:100,000 it was raised to 114.4 ± 1.4 percent of control ($p < 0.05$). This increase in adherence was observed even at the extremely high dilution of 1:1,000,000, where the adherence percentage was 118.1 ± 3.3 of control ($p < 0.05$).

With glutaraldehyde, no lysis of cells was apparent even at a high concentration of 1:10. However, neutrophil adherence was depressed markedly to 0 percent

Table 4.1 Comparative Effects of Pulpotomy Medicaments on PMN Adherence

Pulpotomy Medication Dilution	PMN Adherence (Percent of Control \pm S.D.)			Calcium Hydroxide (Calyx1)
	Formocresol (Buckley's)	Glutaraldehyde	Eugenol	
1:10	lysis	0 †	lysis	lysis
1:100	lysis	45.3 \pm 3.4†	lysis	lysis
1:1000	lysis	108.6 \pm 2.4	lysis	122.7 \pm 3.0†
1:10,000	41.5 \pm 7.5**	104.8 \pm 0.4	88.2 \pm 5.9	116.7 \pm 2.9
1:100,000	114.4 \pm 1.4*	N.D.	121.3 \pm 3.8*	107.6 \pm 9.5
1:1,000,000	118.1 \pm 3.3*	N.D.	121.2 \pm 5.09*	103.2 \pm 7.8

Incubation time with PMNs was 15 minutes for all medicaments while contact time with the nylon fibres was 5 minutes.

N.D. = not done

** p < 0.01

* p < 0.05

† p < 0.02

‡ p < 0.001

compared to control values. At the next dilution of 1:100, neutrophil adherence still was depressed at 45.3 ± 3.4 percent of control ($p < 0.02$). In contrast, at an intermediate concentration of 1:1,000, neutrophil adherence was increased slightly (108.6 ± 2.4), but this increase was not statistically significant ($p > 0.1$). However, at a low concentration of 1:10,000, there was no significant change in neutrophil adherence compared to controls.

Eugenol at high concentrations of 1:10, 1:100, and 1:1,000 caused lysis of neutrophils. At the very low concentrations of 1:100,000 and 1:1,000,000, there was a stimulation of neutrophil adherence. Percentage of neutrophil adherence was 121.3 ± 3.8 of control ($p < 0.05$) at 1:100,000 dilution; and 121.1 ± 5.1 of control at 1:1,000,000 dilution.

Calcium hydroxide at high concentrations of 1:10 and 1:100 produced lysis of neutrophils. At an intermediate concentration of 1:1,000, stimulation of neutrophil adherence was observed at 122.7 ± 3.0 percent of control ($p < 0.02$). At the lower concentration of 1:100,000, no significant effect compared to control was noted.

Effects of prolonged incubation with low concentrations of medicaments

The previous sets of experiments indicated that low concentrations of formocresol, eugenol, and calcium hydroxide caused stimulation of neutrophil adherence. Initial stimulation followed by depression is a well-known response of neutrophils following activation by various stimuli (Boxer et al, 1980). To determine if this activation-deactivation phenomenon is evident upon stimulation with pulpotomy medicaments, concentrations of medicaments producing stimulatory effects on neutrophil adherence were selected and incubated with neutrophils for varying time periods. Figure 4.1 shows that the activation-deactivation phenomenon was observed clearly with formocresol, eugenol, and calcium hydroxide.

Effects of of Formocresol and its constituents on neutrophil adherence

Since formocresol is composed of 19% formaldehyde and 35% cresol, it is pertinent to determine the individual effects of each of these components. Stock solutions of 19% formaldehyde and 35% cresol were made by using medium 199 and absolute ethanol, respectively.

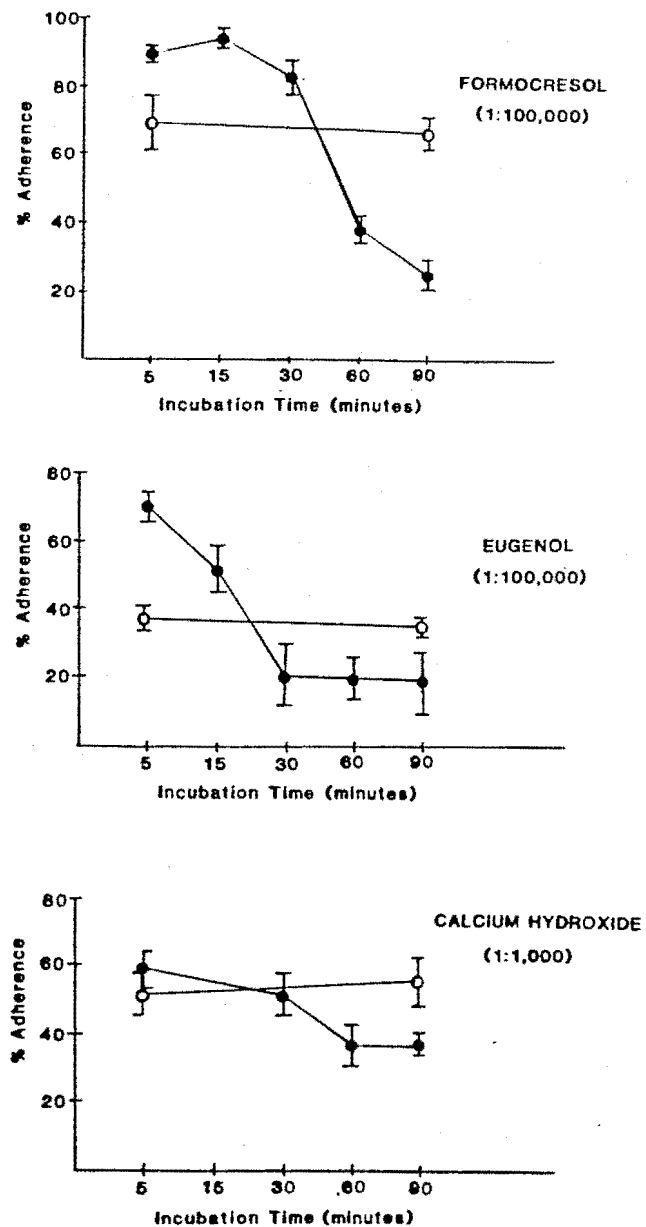


Figure 4.1 Effect of prolonged incubation time with high dilutions of pulpotomy medicaments on PMN adherence. The effect of each medicament was studied with PMNs obtained from a different donor. Experiments were performed in triplicate (mean \pm S.D.), and results shown as closed circles (medicament treated) and open circles (untreated PMNs).

These solutions were diluted further in medium 199 to obtain concentrations of 1:10,000. Solutions of formaldehyde, cresol and formocresol, all at a concentration of 1:10,000 and appropriate controls, with and without ethanol, were incubated with neutrophils at 37°C for 15 min. (Fig. 4.2). Formocresol and formaldehyde at similar dilutions resulted in a comparable decline in neutrophil adherence. In contrast, cresol alone did not alter neutrophil adherence. It was necessary to use alcohol as a solvent in these experiments, but alcohol at this low concentration did not alter neutrophil adherence. Thus, it is the formaldehyde component of the formocresol that is responsible for the effect on neutrophil adherence.

Effect of medicaments on Phorbol Myristate Acetate (PMA)-stimulated neutrophils

Phorbol myristate acetate is a derivative of croton oil with stimulatory effects on immune cells, including neutrophils (Repine et al, 1974). To determine if neutrophils treated with inhibitory concentrations of pulpotomy medicaments could respond to PMA stimulation, formocresol at 1:10,000, eugenol at 1:1,000, and glutaraldehyde at 1:100 dilutions were used in the next set of experiments. Neutrophils first were incubated with PMA (0.01 ug/ml) for 5 min. and then for another 15 min. after the addition of medicament. Appropriate

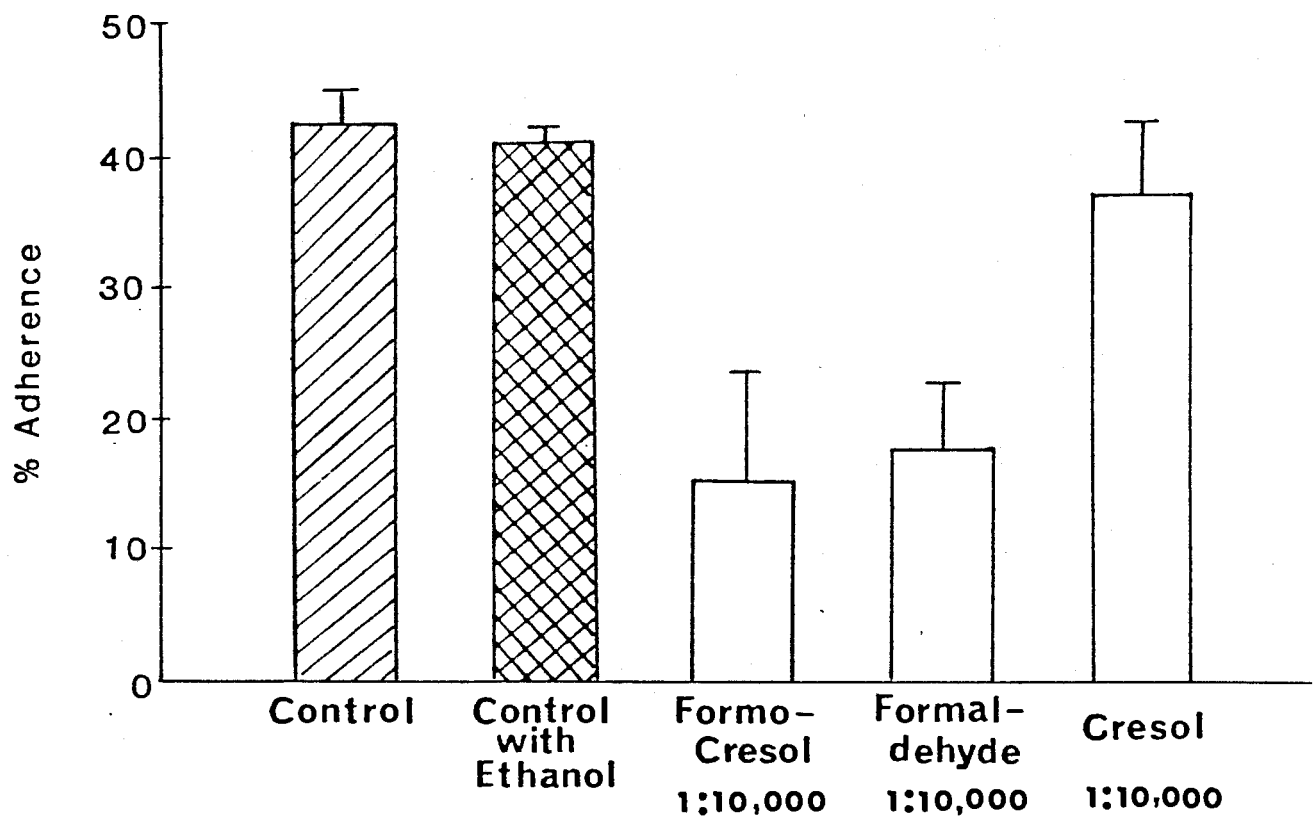


Figure 4.2 Effects of formocresol and its constituents on PMN adherence. Experiments were performed in triplicate and results shown as mean \pm S.D.

controls without PMA also were included in this set of experiments. The results are shown in Figure 4.3. PMA increased the percentage of neutrophil adherence. In contrast, no significant increase in neutrophil adherence was observed in the presence of formocresol at a concentration of 1:10,000. Similar trends were observed in separate sets of experiments using eugenol at 1:1,000 and glutaraldehyde at 1:100.

In these experiments, PMA stimulated the percentage of neutrophil adherence significantly in the controls, but failed to do so in the presence of the pulpotomy medicaments.

Viability studies

To exclude the possibility that alteration of neutrophil adherence is due to loss of cellular viability, the trypan blue dye exclusion studies were performed on neutrophils with medicaments at high and low concentrations. For each medicament concentration, viability counts were determined after incubation periods of 15 min. and 90 min. The results indicated that >97% of neutrophils were viable in all cases even after prolonged incubation with the medicaments (data not presented).

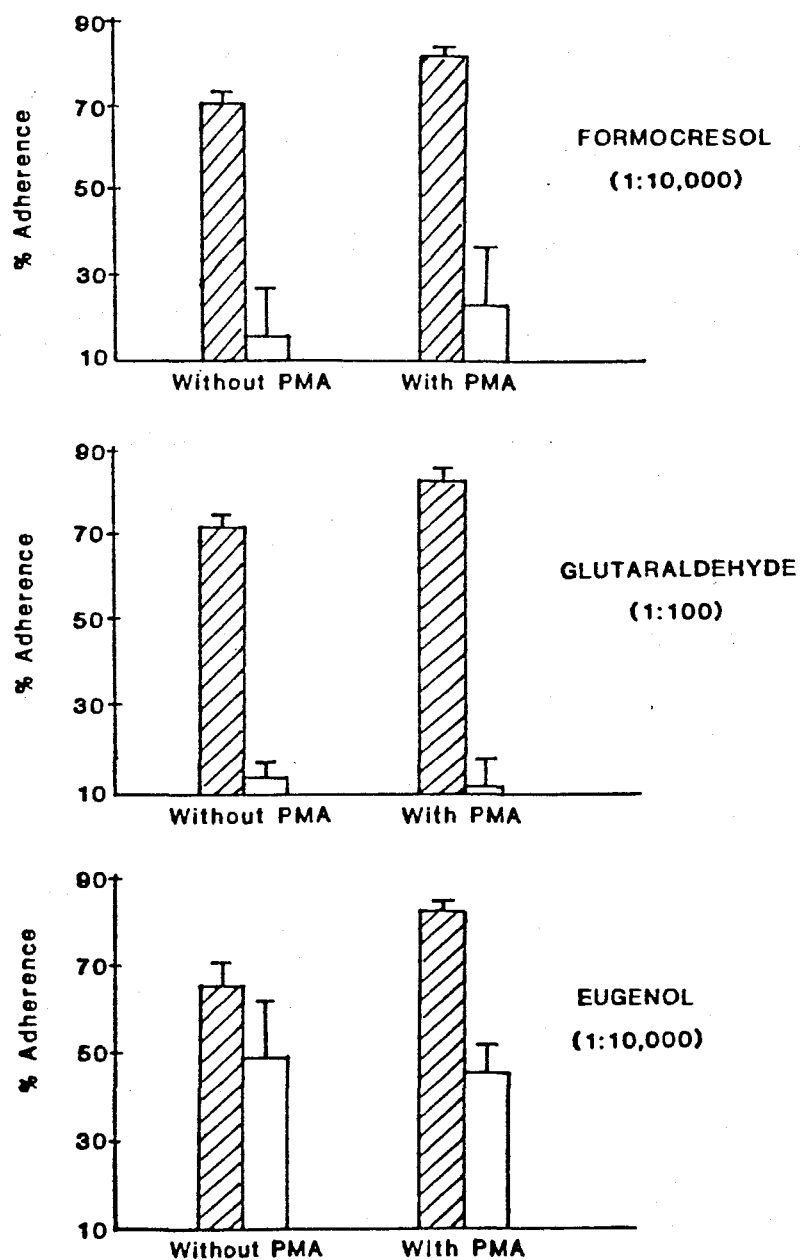


Figure 4.3 Effects of pulpotomy medicaments on PMA-stimulated PMNs. The effect of each medicament was studied with PMNs obtained from a different donor. Experiments were performed in triplicate (mean \pm S.D.), and results shown as open columns (medicament treated) and closed columns (untreated PMNs).

Discussion

The results of the present studies on the effects of pulpotomy medicaments on neutrophil adherence demonstrate a clear correlation with recognised histologic changes seen with the use of these medicaments. With formocresol as the pulpotomy medicament, a zone of fixation usually is evident where the pulp is in direct contact with the medicament. Farther away, where the concentration of formocresol is decreased, there is a zone of poor cellular definition and necrosis. Apical to this is a zone of chronic inflammation which blends into normal tissue (Rolling & Lambjerg-Hansen, 1978). Histologic sections of teeth treated with calcium hydroxide or eugenol also show a zone of tissue necrosis adjacent to these medicaments followed by a zone of chronic inflammation apically (Magnusson, 1970; Magnusson, 1971). In contrast, glutaraldehyde produces a zone of tissue fixation where it is in direct contact with the pulp, while apical to this is a zone of normal tissue with few inflammatory cells (Tagger & Tagger, 1984; Kopel et al, 1980).

In the present studies, lysis of neutrophils was observed with high concentrations of formocresol, eugenol and calcium hydroxide but not glutaraldehyde.

Of greater interest is the finding that low concentrations of formocresol, eugenol, and calcium hydroxide, but not glutaraldehyde, produced significant stimulation of neutrophil adherence. This finding corresponds well to the histological observations of inflammatory changes in the apical zones of the pulp after use of these three medicaments, where the concentrations of the medicaments are low. Stimulation of neutrophils results in increased adherence, followed by diapedesis and migration of these cells to the inflammation site, where they release toxic oxygen free-radicals and lysosomal enzymes (Weissman et al, 1980). The resultant tissue damage, and the persistence of these medicaments around the pulp, would lead to the development of chronic inflammation in the pulp and subsequent tooth loss. In this regard, others have shown that pulp tissue altered by formocresol evoked a specific immune response, both humoral (Block et al, 1978) and cell-mediated (Thode-van Velzen, 1977), and this also may contribute to the chronic inflammatory changes following the use of this medicament.

CHAPTER FIVE

DIRECT MODULATION OF HUMAN NEUTROPHIL ADHERENCE
BY COAGGREGATING PERIODONTOPATHIC BACTERIA

Introduction

Periodontitis, a chronic inflammatory disease of the tooth supporting tissues, is by far the commonest cause of tooth loss in adults. Although dental plaque with its microbial constituents has been firmly implicated as the aetiologic agent in periodontitis (Loe et al, 1965; Lang et al, 1973; Loesche & Syed, 1978; van Palenstein Helderman, 1981), many features of the disease cannot be explained on the basis of microbial factors alone (Page & Schroeder, 1976). Existing theories of immunopathogenesis are based on classical mechanisms such as immune complexes (Brandtzaeg & Kraus, 1965; Ivanyi & Lehner, 1970), complement activation (Allison et al, 1976), lymphocytes (Nisengard, 1977; Seymour et al, 1979) and various combinations of the above (Horton et al, 1974; Page & Schroeder, 1976), and may adequately account for the established and advanced lesions of periodontitis.

However, it is unlikely that classical immunological mechanisms have a major role in the pathogenesis of the early lesions of periodontitis because plaque bacteria are not found in the periodontal tissues until late stages of the disease (Socransky, 1970; Saglie et al, 1982). Moreover, humoral factors are present in only small quantities in gingival fluid, and their role in pathogenesis has been questioned (Clagett & Page, 1978; Hsu & Cole, 1985). Of greater biological relevance are polymorphonuclear neutrophils (PMNs) which comprise over 90% of the leukocytes in gingival fluid (Attstrom & Egelberg, 1970; Cimasoni, 1983). Thus, the direct interaction between plaque bacteria and neutrophils in the micro-environment of the gingival sulcus may hold the key to understanding the immunopathogenesis of periodontitis in general, and the initiation of inflammation in particular.

In this study, we examined the direct interaction between neutrophils and 3 periodontopathic bacteria singly and in combination. The resultant change in neutrophil behaviour is measured with the adherence microassay developed in our laboratory (Thong & Currell, 1983; Seow & Thong, 1986a). Adherence is one of the earliest observable and crucial changes in neutrophil behaviour following neutrophil activation (Gallin, 1985).

Materials and Methods

Bacteria

The bacteria selected for study consists of two Gram negative pathogens which are implicated in the destructive forms of periodontal disease: *Bacteroides gingivalis* (Slots, 1977; Tanner et al, 1979; White & Maynard, 1981) and *Fusobacterium nucleatum* (Tanner et al, 1979). In addition, a Gram positive organism, *Actinomyces viscosus* (Ellen, 1982) also found in subgingival plaque but usually associated with mild periodontal disease was also tested. *F. nucleatum* (strain 263), *B. gingivalis* (ATCC 33277) and *A. viscosus* (T14 Vi) were gifts from Dr A. Tanner (Forsyth Dental Centre, Boston, Massachusetts), Dr D. Love (University of Sydney, New South Wales, Australia), and Dr A. Rogers (University of Adelaide, South Australia), respectively.

F. nucleatum and *A. viscosus* were grown anaerobically in mycoplasma broth (Gibco Diagnostics, Madison, Wisconsin) supplemented with glucose and hemin, with the addition of menadione in the case of *F. nucleatum*. *B. gingivalis* was grown anaerobically in cooked meat media. The purity of the cultures was checked in each case by noting colony morphology on blood agar plates and by Gram staining. The bacteria were harvested by centrifugation at 10,000 g for 15 mins. at 4°C, washed twice in

phosphate buffered saline, and resuspended in medium 199. Cell concentrations were determined by diluting the stock bacterial suspension and counting in a Neubauer haemocytometer.

Neutrophil adherence

Heparinised blood was obtained from healthy donors by venepuncture. Neutrophils were purified by a one-step centrifugation procedure on Mono-Poly resolving medium (Flow Laboratories) as previously described (Ferrante & Thong, 1980). The neutrophils were harvested from the second band, washed twice and resuspended in medium 199. They were of >97% purity.

The neutrophil adherence assay was performed using nylon fibre microcolumns previously described (Thong & Currell, 1983; Seow & Thong, 1986). Briefly, the nylon fibre microcolumns were prepared by carefully weighing out 10mg lots of teased nylon fibre (Olympic Products, Queensland, Australia). These were placed into 100 μ l disposable pipette tips (Stockwell Scientific, California), so as to occupy the centre 2cm portion of the 5cm pipette tip. Neutrophil suspensions, with or without bacteria, were adjusted to concentrations between $4-6 \times 10^6$ cells/ml and 100 μ l was delivered into each nylon fibre microcolumn. After incubation for 5 min. at 37°C and high humidity in order to allow for contact between

neutrophils and nylon fibre, the microcolumns were placed in a specially designed apparatus. The fluid was extracted by a vacuum suction pressure of approximately 250 mbar applied for 2 mins. into disposable test tubes. The concentration of neutrophils was determined by the Neubauer haemocytometer and the results calculated as follows:

$$\text{Percent adherence} = 100 - \frac{\text{CE}}{\text{CA}} \times 100$$

where CA is the concentration of neutrophils applied and CE is the concentration of cells recovered in the effluent. In some experiments, the results were further expressed as percent of control and calculated as follows:

$$\% \text{ of control} = \frac{\% \text{ adherence of test sample}}{\% \text{ adherence of control sample}} \times 100$$

Each experiment was performed in triplicate and the results expressed as mean \pm SE.

Statistical analysis

The student's 't' test was used for statistical analysis of the data.

Results

Effect of varying incubation times

Figure 5.1 shows the effects of incubating the bacteria with neutrophils for 2, 5 and 15 min. at a bacteria:neutrophil ratio of 10:1. It is evident that neutrophil adherence was greatly increased to 140.0 ± 6.9 percent of control ($p < 0.01$) after a very short incubation period of 2 mins for *F. nucleatum*. In contrast *B. gingivalis* and *A. viscosus* caused a marked depression of neutrophil adherence at 2 min. being 74.1 ± 3.2 percent of control ($p < 0.05$) and 31.9 ± 1.4 ($p < 0.001$) percent of control, respectively.

At 5 min. neutrophil adherence was increased to 134.9 ± 3.7 percent of control ($p < 0.01$) for *F. nucleatum*. With *B. gingivalis* and *A. viscosus*, neutrophil adherence continued to be depressed at 71.7 ± 4.7 percent of control ($p < 0.05$) and 36.8 ± 2.3 percent of control ($p < 0.05$), respectively.

After 15 mins. incubation, the modulatory effects by bacteria on neutrophil adherence appeared to wane, most likely due to neutrophil exhaustion in this in vitro system.

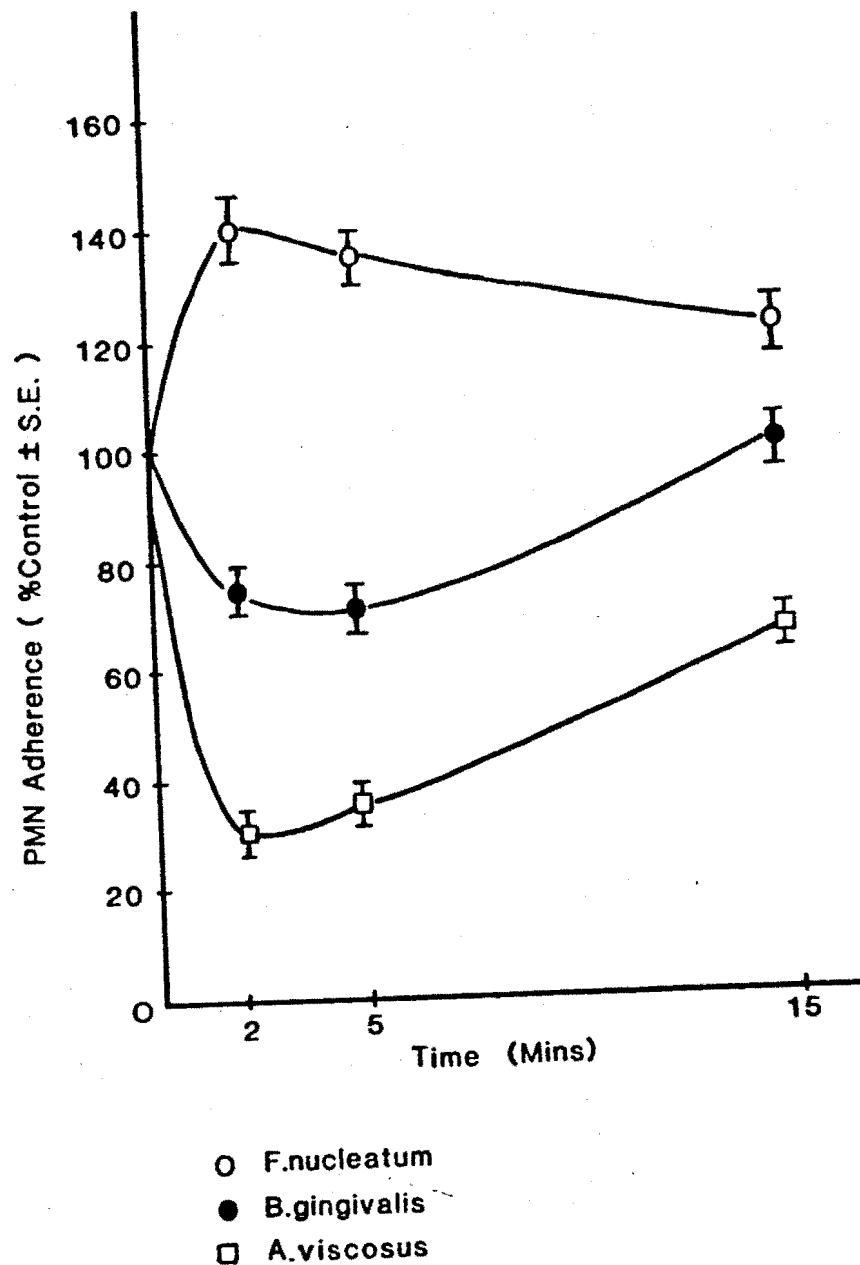


Figure 5.1 Effects of varying incubation times with oral bacteria on PMN adherence. Values were expressed as percentages of control (no bacteria) which is represented as 100% adherence. Actual PMN adherence of control varied from 40-60% (data not presented).

Percent of control is calculated as percent adherence of test sample $\times 100$ /percent adherence of control sample. Experiments were performed in triplicate and results expressed as mean \pm S.E.

Effect of varying bacteria:neutrophil ratios

To determine optimum bacteria numbers on neutrophil adherence, suspensions with bacteria:neutrophil ratios of 10:1, 1:1 and 1:10 were prepared and incubated at 37°C for 5 min. (Table 5.1). At a bacteria:neutrophil ratio 10:1, *F. nucleatum* increased neutrophil adherence to 148.5 ± 9.0 ($p < 0.05$) percent of control. In contrast, *B. gingivalis* and *A. viscosus* markedly depressed neutrophil adherence to 62.1 ± 1.4 ($p < 0.01$) and 54.1 ± 2.7 ($p < 0.01$) percent of control, respectively.

At a bacteria:neutrophil ratio of 1:1, percent neutrophil adherence was still increased for *F. nucleatum* (124.5 ± 5.0 , $p < 0.05$) compared to control, and decreased for *B. gingivalis* (66.5 ± 2.0 , $p < 0.05$) and *A. viscosus* (66.7 ± 2.3 , $p < 0.05$).

When the concentrations of bacteria were further diluted to obtain the bacteria:neutrophil ratio of 1:10, the modulatory effects of bacteria on neutrophil adherence were no longer evident. In all cases, neutrophil adherence values were not significantly different from control ($p > 0.1$). The above results were confirmed by a second experiment (Table 5.1)

Table 5.1 Effects of incubation with varying bacteria:PMN ratio on PMN adherence

Bacteria:PMN ratio	Mean PMN adherence (% of control \pm S.E.)		
	F.n.	B.g.	A.v.
Experiment 1			
10:1	148.5 \pm 9.0*	62.1 \pm 1.4**	54.1 \pm 2.7**
1:1	124.5 \pm 3.0*	66.5 \pm 1.0*	66.7 \pm 2.3**
1:10	96.0 \pm 2.9	85.0 \pm 3.9	93.9 \pm 5.9
Experiment 2			
10:1	139.2 \pm 3.5**	71.6 \pm 4.7*	51.1 \pm 5.3**
1:1	118.9 \pm 2.2**	74.6 \pm 2.5*	69.3 \pm 2.1***
1:10	104.3 \pm 3.0	80.0 \pm 3.1	96.7 \pm 3.4

Each experiment was performed with PMNs obtained from different donors. PMNs were incubated with varying concentrations of bacteria for 5 min at 37°C prior to adherence microassay. Results were expressed as mean \pm S.E. of triplicate samples.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

+ Actual PMN adherence of control (no bacteria) varied from 40-60% (data not presented). Percent of control is calculated as percent adherence of test sample \times 100/percent adherence of control sample.

Treatment of bacteria with physical and chemical agents

Figure 5.2 shows the results of experiments on the effects of various physical and chemical treatments of bacteria on neutrophil adherence. In the first set of experiments using heat-treated bacteria (75°C for 60 mins), it can be observed that untreated *F. nucleatum* increased percent neutrophil adherence from 36.4 ± 1.6 to 67.6 ± 4.1 ($p < 0.05$). After heat treatment, percent neutrophil adherence fell to 25.4 ± 1.1 ($p < 0.01$) showing a reversal of the original stimulation.

With *B. gingivalis* and *A. viscosus*, untreated bacteria depressed percent neutrophil adherence from 36.4 ± 1.6 in control to 20.6 ± 1.0 ($p < 0.02$) in the case of *B. gingivalis* and from 36.4 ± 1.6 in control to 24.3 ± 4.4 ($p < 0.05$) in the case of *A. viscosus*. Heat-treatment significantly reversed the inhibitory effects of these 2 bacteria.

Formalin

Treatment of bacteria with 2% formalin for 24 hours at 37°C resulted in marked changes in neutrophil adherence as shown in Figure 5.2. Untreated *F. nucleatum* again increased percent neutrophil adherence from 52.5 ± 3.0 seen in controls to 74.8 ± 5.3 ($p < 0.05$). However, after formalin treatment, percent neutrophil adherence fell to 38.6 ± 3.1 .

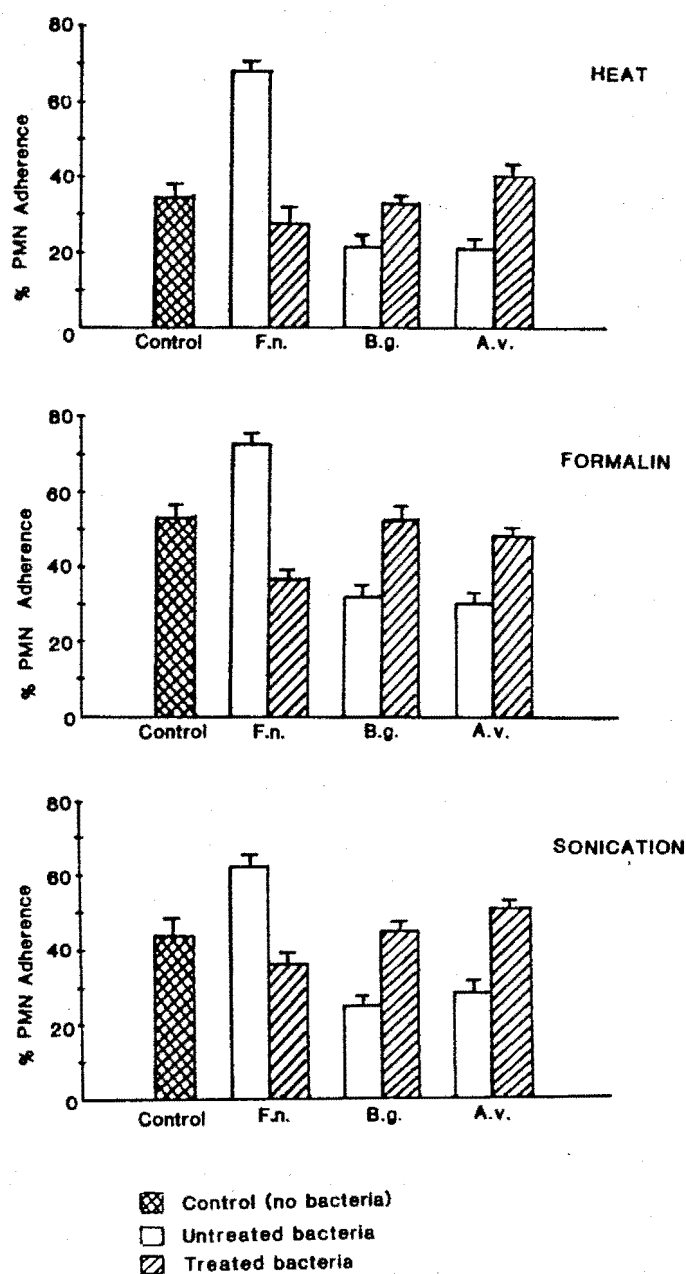


Figure 5.2 Treatment of bacteria with physical and chemical agents. In all experiments, bacteria:PMN ratios of 10:1 and incubation times of 5 min were used. Experiments were performed in triplicate and results expressed as mean \pm S.E.

With *B. gingivalis* and *A. viscosus*, untreated bacteria depressed percent neutrophil adherence from 52.5 ± 3.0 in control to 32.6 ± 3.0 ($p < 0.02$) in the case of *B. gingivalis* and to 35.1 ± 4.4 ($p < 0.05$) in the case of *A. viscosus*. In contrast, after treatment with formalin, percent neutrophil adherence was not significantly different from control in both bacteria.

Sonication

Bacteria at concentrations of $50 \times 10^6/\text{ml}$ were sonicated in an ice bath using a Branson sonicator with probe tip set at 6. Sonication was achieved by applying intermittent bursts of 15 seconds for 3 hours. Microscopy of the sonicated preparations revealed 80% lysis of bacteria. The sonicated preparations were centrifuged at 2000g for 10 mins. which sedimented any remaining whole cells. The resulting supernatant fluid was used for the neutrophil adherence assay.

The effects of sonication of the bacteria on neutrophil adherence are shown in Figure 5.2. In the case of *F. nucleatum*, untreated bacteria again increased percent neutrophil adherence to 61.9 ± 2.5 compared to 44.2 ± 3.8 in controls ($p < 0.001$). In contrast, after sonication, neutrophil adherence fell back to control values.

In the case of *B. gingivalis*, untreated bacteria decreased percent neutrophil adherence to 26.0 ± 4.5 ($p < 0.02$). After sonication, percent neutrophil adherence increased to a value comparable to control (45.4 ± 2.9 , $p > 0.1$). Similar trends were observed with *A. viscosus*. In this case, untreated bacteria caused a depression of percent neutrophil adherence to 29.5 ± 4.1 ($p < 0.05$) compared with the control value of 45.4 ± 2.9 . However, after sonication neutrophil adherence was increased to 52.8 ± 1.1 ($p < 0.05$).

Effects of incubation with monosaccharides

As the interaction of bacteria with neutrophils may involve specific carbohydrate receptors (Jones & Freter, 1976; Ofek et al, 1977), we examined possible inhibitory effects of specific monosaccharides on the modulation of neutrophil adherence by bacteria. D-galactose (Sigma Co., St Louis, Missouri) was selected because it has been shown to block the haemoagglutinating activity of *F. nucleatum* (Mongiello & Falker, 1979). To establish specificity of the reaction, the effects of another 6-carbon sugar, D-glucose was also examined. These sugars were used at a concentration of 100mM, which is optimum for inhibition of haemagglutination by *F. nucleatum* (Mongiello & Falker, 1979). Bacterial suspensions were mixed with D-glucose and D-galactose respectively and

incubated for 15 min. before addition of neutrophil suspensions.

Figure 5.3 shows the results of experiments using D-galactose and D-glucose to block the effects of bacteria. In the case of *F. nucleatum*, untreated bacteria increased percent neutrophil adherence to 72.5 ± 2.1 compared with 56.9 ± 5.5 in control ($p < 0.02$). However, after incubation with D-galactose, percent neutrophil adherence fell to control levels (55.3 ± 2.3 , $p > 0.1$), showing that D-galactose but not D-glucose could block the stimulatory effects of *F. nucleatum* on neutrophil adherence.

In the case of *B. gingivalis*, untreated bacteria resulted in significant depression of neutrophil adherence compared to control (28.1 ± 2.5 , vs 46.6 ± 2.5 , $p < 0.01$). Treatment of bacteria with D-galactose but not D-glucose abrogated this effect.

In contrast, different trends were observed with *A. viscosus*. Untreated *A. viscosus* depressed percent neutrophil adherence to 29.9 ± 4.5 ($p < 0.01$) compared to a control value of 57.5 ± 5.0 . Neither galactose nor glucose altered the depression of neutrophils seen with untreated bacteria.

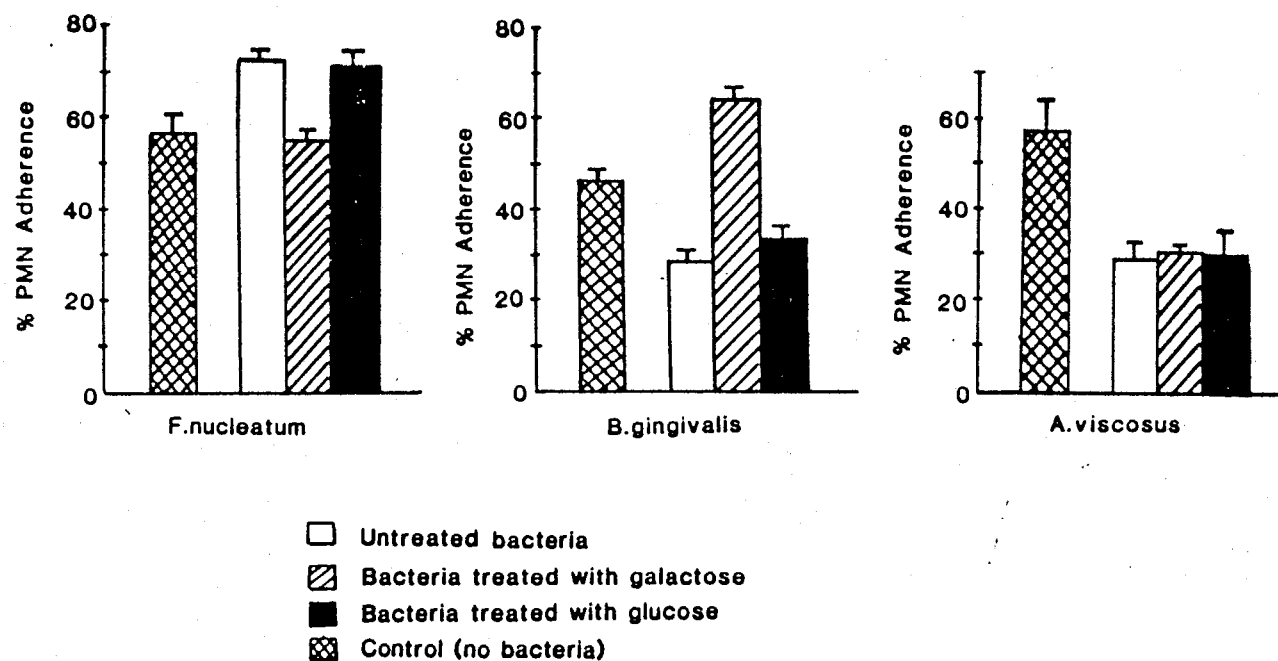


Figure 5.3 Effects of incubation with monosaccharides. Bacteria were incubated with 100 mM of sugars for 15 min then mixed with PMNs for 5 min prior to PMN adherence assay. Each experiment was performed with PMNs obtained from different donors. The experiments were performed in triplicate and the results expressed as mean \pm S.E.

The above results indicate that the modulation on neutrophil adherence by *F. nucleatum* and *B. gingivalis* involve specific receptors on the cell surface and that these receptors are blocked by D-galactose.

Effects of bacterial coaggregates on neutrophil adherence

Coaggregation between pairs of bacteria were achieved by a method previously described (Cisar et al, 1979). Briefly, equal volumes (0.2 ml) of bacterial suspensions (about 5×10^9 cells/ml) were mixed vigorously for 10 sec. on a Vortex mixer, allowed to stand at room temperature for 1 hour, mixed again and scored for coaggregation. Scores for the degree of coaggregation ranged from "zero" to "four plus" and were assigned by the following criteria: zero - no visible coaggregates in the cell suspension; plus one - small uniform coaggregates in suspension; plus two - definite coaggregates easily seen but suspension remained turbid without immediate settling of coaggregate; plus three - large coaggregates which settled rapidly leaving some turbidity in the supernatant fluid; plus four - clear supernatant fluid.

i) Coaggregation of *F. nucleatum* and *B. gingivalis*

When equal concentrations of *F. nucleatum* and *B. gingivalis* were mixed together the coaggregation effect

was scored as +1 (small uniform aggregates in suspension). Microscopy confirmed over 80% of bacteria coaggregated. As shown in Table 5.2, when these coaggregates of bacteria were mixed with neutrophils, there was an increase in percent neutrophil adherence over that of control (72.7 ± 1.2 vs 52.1 ± 1.3 , $p < 0.01$). This increase in percent neutrophil adherence was similar to that seen with *F. nucleatum* alone (71.5 ± 2.6 , $p < 0.01$). In contrast, *B. gingivalis* alone depressed percent neutrophil adherence to only 35.7 ± 3.2 ($p < 0.01$). These results indicate that when *F. nucleatum* and *B. gingivalis* are coaggregated, the effects of *F. nucleatum* appear to dominate the reaction with neutrophils.

ii) Coaggregation of *F. nucleatum* and *A. viscosus*

Mixture of equal concentrations of *F. nucleatum* and *A. viscosus* resulted in coaggregates which were scored as +2 (definite coaggregates easily seen but suspensions remaining turbid). Microscopy confirmed that over 80% of bacteria coaggregated. *F. nucleatum* alone increased percent neutrophil adherence to 70.0 ± 3.9 ($p < 0.05$) compared to a control value 54.8 ± 2.9 (Table 5.2). In contrast, *A. viscosus* depressed percent neutrophil adherence to 39.4 ± 5.1 ($p < 0.05$). However, when *F. nucleatum* and *A. viscosus* were mixed together, percent neutrophil adherence was increased to 65.5 ± 1.3 ($p <$

Table 5.2 Effects of bacterial coaggregates on PMN adherence

Coaggregating bacteria	% PMN Adherence (Mean \pm S.E.)
<u>Exp. 1</u>	
F. nucleatum	71.5 \pm 2.6**
B. gingivalis	35.7 \pm 3.2**
F. nucleatum + B. gingivalis	72.7 \pm 1.2**
Control (no bacteria)	52.1 \pm 1.3
<u>Exp. 2</u>	
F. nucleatum	70.0 \pm 3.9*
A. viscosus	39.4 \pm 5.1*
F. nucleatum + A. viscosus	65.5 \pm 1.3**
Control (no bacteria)	54.8 \pm 2.9
<u>Exp. 3</u>	
B. gingivalis	33.6 \pm 3.2*
A. viscosus	35.8 \pm 1.0*
B. gingivalis + A. viscosus	56.7 \pm 1.4
Control (no bacteria)	50.3 \pm 4.3
<u>Exp. 4</u>	
F. nucleatum + A. viscosus + B. gingivalis	67.8 \pm 0.9**
Control (no bacteria)	53.9 \pm 1.4

Each experiment was performed with PMNs obtained from different donors. PMNs were incubated with coaggregated bacteria at bacteria: PMN ratios of 10:1 for 5 min at 37°C prior to adherence microassay. Results were expressed as mean \pm S.E. of triplicate samples.

* $p < 0.05$

** $p < 0.01$

0.01), again showing the dominance of *F. nucleatum* in the interaction of the coaggregated bacteria on neutrophil adherence.

iii) Coaggregation of *B. gingivalis* and *A. viscosus*

When equal concentrations of *B. gingivalis* and *A. viscosus* were mixed, coaggregates resulted which were scored at +1. As shown in Table 5.2, *B. gingivalis* alone depressed percent neutrophil adherence to 33.6 ± 3.2 compared to a control value of 50.3 ± 4.3 ($p < 0.05$), and *A. viscosus* depressed percent neutrophil adherence to a comparable value of 35.8 ± 1.0 . In contrast, when the two bacteria were coaggregated, percent neutrophil adherence was comparable to control (56.7 ± 1.4 vs 50.3 ± 4.3 , $p > 0.1$).

iv) Coaggregation of *F. nucleatum*, *A. viscosus*, *B. gingivalis*

When these three bacteria were mixed together in equal concentrations, a coaggregation score of +2 resulted. The coaggregates caused an increase in percent neutrophil adherence to 67.8 ± 0.9 ($p < 0.01$) compared to a control value of 53.9 ± 1.4 . These results again indicate the dominant effect of *F. nucleatum* in the interaction of the bacterial coaggregates with neutrophils.

v) Effects of bacterial coaggregates and galactose on neutrophil adherence

When bacterial coaggregates were mixed with 100 mM galactose, their effects on neutrophil adherence appeared to be blocked. As shown in Table 5.3, when coaggregates of *F. nucleatum* and *B. gingivalis* were incubated with neutrophils for 5 mins, without galactose, percent neutrophil adherence was increased to 66.9 ± 1.5 compared to 44.8 ± 3.2 in control ($p < 0.01$). In contrast, after incubation with galactose, neutrophil adherence was not significantly different from control.

Coaggregates of *F. nucleatum* and *A. viscosus* showed similar results. Without galactose, these coaggregates increased percent neutrophil adherence to 63.9 ± 3.2 , compared to 45.4 ± 1.9 in control ($p < 0.01$). However, in the presence of galactose, percent neutrophil adherence was not significantly different from control.

Similarly, coaggregates of *F. nucleatum*, *A. viscosus* and *B. gingivalis* increased percent neutrophil adherence compared to control in the absence of galactose (60.2 ± 2.1 vs 47.0 ± 2.4 , $p < 0.01$). In contrast, when galactose was added, percent neutrophil adherence became comparable to control.

Table 5.3 Effects of incubating bacterial coaggregates with galactose on PMN adherence

Coaggregating bacteria	% PMN Adherence (Mean \pm S.E.)		
	Without galactose	With galactose	Control (no bacteria)
F. nucleatum			
+ B. gingivalis	66.9 \pm 1.5*	40.3 \pm 1.2	44.8 \pm 3.2
F. nucleatum			
+ A. viscosus	63.9 \pm 3.2*	48.1 \pm 2.2	45.4 \pm 2.9
F. nucleatum			
+ A. viscosus			
+ B. gingivalis	60.2 \pm 2.1*	48.8 \pm 1.0	47.0 \pm 2.4

Each experiment was performed with PMNs obtained from different donors. Coaggregated bacteria were incubated with 100 mM glucose or galactose for 15 min, then mixed with PMNs for a further 5 min prior to PMN adherence assay. Bacteria:PMN ratios used were 10:1. Results were expressed as mean \pm S.E. of triplicate samples.

* $p < 0.01$

The above results support the earlier observation that effects of *F. nucleatum* are dominant in the reaction of bacterial coaggregates. When its receptors were blocked with galactose, the stimulatory effects on neutrophil adherence were no longer evident.

Discussion

Although humoral factors are present in gingival fluid, they are present in small concentrations, and their relevance to the pathogenesis of early inflammatory changes in periodontitis have not been substantiated (Clagett & Page, 1978; Hsu & Cole, 1985). Yet most studies concerning the interaction between plaque bacteria and neutrophils focussed on the intermediation of immunoglobulin and complement. Only a few investigators have studied the direct interaction between bacteria and neutrophils as part of larger studies involving serum components, and then not in a systematic fashion. In this regard, Tsai et al (1978) and Taichman et al (1984) showed that *F. nucleatum*, more so than *A. viscosus* or *B. gingivalis*, directly induced the release of lysosomal enzymes by neutrophils. More recently, Passo et al (1982) demonstrated that *F. nucleatum* but not *B. gingivalis*, enhanced neutrophil chemiluminescence.

The results of the present study confirm and extend these previous observations. We found that *F. nucleatum* enhanced neutrophil adherence by up to 140% of control values, while *B. gingivalis* and *A. viscosus* depressed neutrophil adherence to 74% and 32% of control values, respectively. Also, alteration of the bacterial cell surface by physico-chemical agents such as heat, formalin and untrasonication resulted in the abrogation of their modulatory effects on neutrophil behaviour. These modulatory effects may be dependent on carbohydrate or glycoprotein molecules because the effects could be blocked by monosaccharides in the medium.

To our knowledge, previous studies have not addressed the issue of bacterial coaggregation and neutrophil behaviour, in spite of the fact that dental plaque is a complex composite of coaggregated oral bacteria. However, studies using freshly isolated plaques have shown that they have profound stimulatory effects on neutrophils (Baehni et al, 1979; Taichman & McArthur, 1976; Taichman et al, 1977). Our data show that *F. nucleatum* may be responsible to a large extent for the stimulatory effects of dental plaque, as all permutations of coaggregates involving this bacteria produced marked neutrophil stimulation.

The mechanism(s) by which direct-interaction between bacteria and neutrophils result in the modulation of neutrophil behaviour is the subject of current research in our laboratory. One possibility may be that the binding of periodontopathic bacteria to neutrophil membrane receptors results in the modulation of expression of glycoprotein molecules (Gallin, 1985) which mediate neutrophil adherence and adherence-dependent functions. Another possibility, which we favour, is that the bacteria-neutrophil interaction leads to the release of neutrophil self-regulatory factors whose biologic roles are that of molecular mediators for recruitment of resting neutrophils so as to amplify immunological and inflammatory responses (Seow & Thong, 1986b).

The findings of the present study allow us to speculate on the early events in the pathogenesis of chronic periodontitis. In the micro-environment of the gingival sulcus where the neutrophils are the predominant leukocytes, and humoral factors are present in small quantities (Clagett & Page, 1978; Hsu & Cole, 1985), the colonizing ability of certain species of oral bacteria may be partially dependent on suppression of neutrophil function by direct bacteria-neutrophil interaction. In this context, *A. viscosus* and *B. gingivalis*, which are shown to have inhibitory effects on neutrophils, are

also successful early colonizers of subgingival plaque (White & Maynard, 1981; Ellen, 1982; Loesche & Syed, 1978; Slots, 1979). The capacity of these bacterial species to escape destruction by neutrophils paves the way for other periodontopathic organisms to invade the periodontal pocket by forming coaggregation bridges to facilitate plaque development (Ellen, 1982; Kolenbrander, 1985). The later presence of *F. nucleatum* and other bacteria with stimulatory effects on neutrophils would cause the release of toxic oxygen radicals and lysosomal enzymes with resultant damage to periodontal tissue (Ishikawa et al, 1982; Taichman & Baehni, 1977; Weissman et al, 1980). Neutrophil-mediated inflammatory damage to the periodontium may be critical for the initiation and sustenance of the immunological reactions seen in this disease. The polymicrobial nature of dental plaque, and the particular mix of the bacterial species, may be responsible for the variations in clinical severity and rate of progress of periodontal lesions.

CHAPTER SIX

**BACTERIA-PHAGOCYTE INTERACTIONS: FUSOBACTERIUM-
INDUCED SECRETION OF A NEUTROPHIL
SELF-REGULATORY FACTOR**

Introduction

Periodontitis is one of the most common diseases found in humans. It affects almost all individuals, and is the major cause of tooth loss in adults. It is characterised by chronic inflammation of the tooth supporting tissues. The microbial aetiology of periodontitis is well recognised but the exact mechanisms of pathogenesis of the chronic inflammatory processes are still unclear (Loe et al, 1965; Lang et al, 1973; Loesche & Syed, 1978; Van Palenstein-Helderman, 1981). Current theories of causation have focused mainly on the later and progressive phases of the disease (Brandtzaeg & Kraus, 1965; Ivanyi & Lehner, 1970; Allison et al, 1976; Page & Schroeder, 1976; Nisengard, 1977; Seymour et al, 1979b).

In the microenvironment of the gingival sulcus, humoral factors are present only in small quantities (Clagett & Page, 1978; Hsu & Cole, 1985), and plaque bacteria come into direct contact with the sulcular leukocytes, of which over 90% are polymorphonuclear neutrophils (Aristrom & Egelberg, 1970; Scully & Challacombe, 1979; Cimasoni, 1983). Since plaque microorganisms are not found in periodontal tissues except in the advanced stages of the disease (Socransky, 1970; Frank, 1980; Saglie et al, 1982), it is unlikely that classical immunological mechanisms have a major role in the initiation of inflammation. Thus, direct interactions between plaque bacteria and neutrophils may have greater biologic relevance for early events in the pathogenesis of periodontitis.

In this paper, we report that incubation of neutrophils with *Fusobacterium nucleatum*, a plaque bacterium implicated in destructive forms of periodontitis, resulted in the secretion of a soluble mediator with stimulatory properties for other neutrophils. The release of this soluble factor(s) after stimulus-specific triggering may represent a major molecular mediator for the recruitment of resting neutrophils, and serve as an amplifying mechanism for the immunological and inflammatory responses.

Materials and Methods

Bacteria

Fusobacterium nucleatum (strain 263) and *Bacteroides gingivalis* (ATCC 33277) were gifts from Dr. A. Tanner (Forsyth Dental Centre, Boston, Massachusetts), and Dr. D. Love (University of Sydney, Australia), respectively. The bacteria were grown anaerobically in mycoplasma broth (Gibco Diagnostics, Madison, Wisconsin), supplemented with glucose, hemin and menadione. The purity of the cultures was checked in each case by noting colony morphology on blood agar plates and by Gram staining. The bacteria were harvested by centrifugation at 10,000g for 15 mins. at 4°C, washed twice in phosphate buffered saline and resuspended in phosphate buffered saline. Cell concentrations were determined by diluting the stock bacterial suspension and counting in a Neubauer haemocytometer.

Neutrophil isolation

Neutrophils were purified from heparinised blood of healthy volunteers using a one-step centrifugation procedure on Mono-Poly resolving medium (Flow Laboratories, Virginia) as previously described (Ferrante & Thong, 1980). The neutrophils were harvested from the second band, washed twice and resuspended in Dulbecco's Balanced Salt Solution (BSS) (Flow Laboratories,

Virginia). They were of >97% purity (Ferrante & Thong, 1980).

Neutrophil adherence microassay

The neutrophil adherence assay was performed using nylon fibre microcolumns as previously described (Thong & Currell, 1983; Seow & Thong, 1986a). Briefly, 10mg lots of teased nylon fibres were packed into 100 μ l disposable pipette tips (Stockwell Scientific, Monterey, California), to occupy the centre 2cm portion of the 5cm pipette tips. The concentrations of neutrophil suspensions were adjusted to $4-6 \times 10^6$ cells/ml and 100 μ l was delivered into each nylon fibre microcolumn. After incubation for 5 mins at 37°C and high humidity in order to allow for contact between neutrophils and nylon fibre, the microcolumns were placed in a specially designed apparatus. Vacuum suction pressure of 250 mbar was applied for 1 min. to extract the fluid from the nylon fibre microcolumns into disposable test tubes. The concentration of neutrophils was determined by the Neubauer haemocytometer and the results calculated as follows:

$$\text{Percent.adherence} = 100 - \frac{\text{CE}}{\text{CA}} \times 100$$

CA

where CE is the

concentration of neutrophils in the effluent and CA is the concentration of neutrophils in the original sample.

Each experiment was performed in triplicate and the results expressed as mean \pm SD.

Deoxyglucose uptake microassay

We used a newly developed deoxyglucose uptake microassay (Seow et al, 1987e) modified from previously described techniques (McCormack et al, 1981). To each well of a round-bottom microtitre plate was delivered approximately 5×10^5 neutrophils in 0.05ml of Dulbecco's BSS. Another 0.1ml of neutrophil supernatant was added to test wells. Control wells received Dulbecco's BSS without neutrophil supernatant. A further 0.05ml of Dulbecco's BSS containing 2D- ^3H -deoxyglucose (Radiochemical Centre, Amersham, UK) was added to each well to give a final concentration of 0.79 $\mu\text{Ci/ml}$. The final volume in all wells was kept constant at 0.2ml. The microtitre plate was incubated at 37°C in a humidified-air atmosphere for 30 mins, centrifuged at 4°C and 800g for 5 mins. and 0.05 ml of supernatant removed for determination of radioactivity in a LKB liquid scintillation counter. The uptake of ^3H -deoxyglucose was calculated from the formula:

Deoxyglucose uptake

$$= \text{Total DPM added} - \text{DPM in supernatant}$$

% Deoxyglucose uptake = $\frac{\text{deoxyglucose uptake}}{\text{Total DPM added}}$

Total DPM added

Experiments were performed in triplicate and the results expressed as mean \pm SD.

Statistical analysis

The student's t-test was used for statistical analysis of the results.

Results

Secretion of soluble factors

In the first set of experiments we examined the modulatory effects of supernatants from bacteria-neutrophil interaction on neutrophil adherence and deoxyglucose uptake. We chose *F. nucleatum* and *B. gingivalis* because these bacteria are strongly implicated in the pathogenesis of periodontitis (Tanner et al, 1979; White & Maynard, 1981). We incubated 5×10^6 neutrophils/ml with 5×10^7 bacteria/ml for 30 mins. at 37°C , and the supernatant obtained by centrifugation at 1000g for 10 mins. The supernatant was passed through 0.2 μm millipore filters to remove any contaminating bacteria. The supernatant was then incubated with neutrophils from a separate donor at a 1:2 dilution. The neutrophil adherence assay was performed after 15 mins. incubation, the ^3H -deoxyglucose uptake was performed after 90 minute incubation. Three sets of controls were used, one containing medium alone, and the

other two containing supernatants from either neutrophil or bacteria alone. The results are presented in Table 6.1. It can be observed that the supernatant from *F. nucleatum*-neutrophil interaction contained a factor which significantly enhanced neutrophil adherence from the baseline of 68.1 ± 6.1 percent to 81.8 ± 1.7 percent ($p < 0.05$), and deoxyglucose uptake from $53,235 \pm 1467$ DPM to $68,315 \pm 2480$ DPM ($p < 0.01$). The supernatant from neutrophils incubated alone or bacteria alone had no significant effects. In contrast, the supernatant from *B. gingivalis*-neutrophil interaction significantly depressed neutrophil adherence from 59.6 ± 2.4 percent to 46.6 ± 5.9 percent ($p < 0.05$), but had no effect on deoxyglucose uptake.

Kinetics of secretion

We next looked at the kinetics of the secretion of the stimulatory factor from *F. nucleatum*-neutrophil interactions. The results (Table 6.2) show that the soluble factor was detectable within 15 mins. of interaction and became maximal at 30 mins. incubation.

Effect of metabolic inhibitors on secretion

In the next set of experiments, we pre-treated neutrophils with cytochalasin B (5 ug/ml) (Sigma, St. Louis, USA), or sodium fluoride (20mM) (May & Baker, Australia) for 60 mins, washed twice in medium before

Table 6.1 Bacteria-neutrophil interactions: modulatory effects of supernatants on neutrophil adherence and deoxyglucose uptake.

Supernatant	F. nucleatum		B. gingivalis	
	% Adherence (mean \pm S.D.)	³ H-deoxyglucose uptake (DPM \pm S.D.)	% Adherence (mean \pm S.D.)	³ H-deoxyglucose uptake (DPM \pm S.D.)
Control (medium)	68.1 \pm 6.1	53,235 \pm 1467	59.6 \pm 2.4	65,254 \pm 1177
Neutrophil alone	68.2 \pm 1.7	51,392 \pm 653	62.5 \pm 2.1	63,994 \pm 587
Bacteria alone	68.2 \pm 2.7	55,568 \pm 1321	63.2 \pm 0.6	67,241 \pm 1733
Bacteria-neutrophil	81.8 \pm 1.7*	68,315 \pm 2840**	46.6 \pm 5.9*	66,115 \pm 1610

Supernatant from each sample was removed after 30 mins, incubated with neutrophils for 90 mins and assayed for ³H-deoxyglucose uptake, or incubated with neutrophils for 15 mins and assayed for adherence.

*p < 0.05

**p < 0.01

Table 6.2 *F. nucleatum*-neutrophil interactions: kinetics of secretion of a soluble factor(s).

Time of bacteria-PMN interaction	³ H-deoxyglucose uptake DPM (mean \pm S.D.)
Control	17,990 \pm 672
15 mins	25,847 \pm 326*
30 mins	27,926 \pm 674*
60 mins	26,242 \pm 744*
90 mins	26,210 \pm 756*

F. nucleatum (5×10^7 /ml) was incubated with neutrophils (5×10^6 /ml) and the supernatant removed at 15, 30, 60 and 90 mins. These samples were added to neutrophils from a separate donor and assayed for ³H-deoxyglucose uptake after 90 mins incubation.

*p < 0.001

interaction with *F. nucleatum*. The results, summarised in Fig. 6.1, show that pre-treatment with cytochalasin B, a potent inhibitor of microfilament function, causes significant suppression of factor secretion ($p < 0.01$). No effect on secretion was seen with sodium fluoride, a known inhibitor of neutrophil oxygen metabolism (Matzner et al, 1982).

Heat and formalin damage to bacteria

To determine whether an intact bacterial surface is required for the specific triggering of factor secretion by neutrophils, we denatured *F. nucleatum* by heat (75°C for 60 mins.), or formalin (2% for 24 hrs. at 37°C), and incubated these treated bacteria with neutrophils in the usual manner. As shown in Figure 6.2, factor secretion by neutrophils was markedly diminished by both processes.

Heat and trypsin treatment of soluble factors

The nature of the neutrophil factor was examined by trypsin and heat treatment. The supernatant obtained from *F. nucleatum*-neutrophil interaction was treated with trypsin (250 µg/ml) (Sigma, St. Louis, USA) for 30 mins. at 27°C followed by antitrypsin (500 µg/ml) (Sigma, St. Louis, USA) for another 30 mins. This treatment markedly reduced the activity of the factor ($p < 0.001$). (Fig. 6.3). Similarly, heating the factor at 56°C for 60 mins. or 80°C for 30 mins. significantly

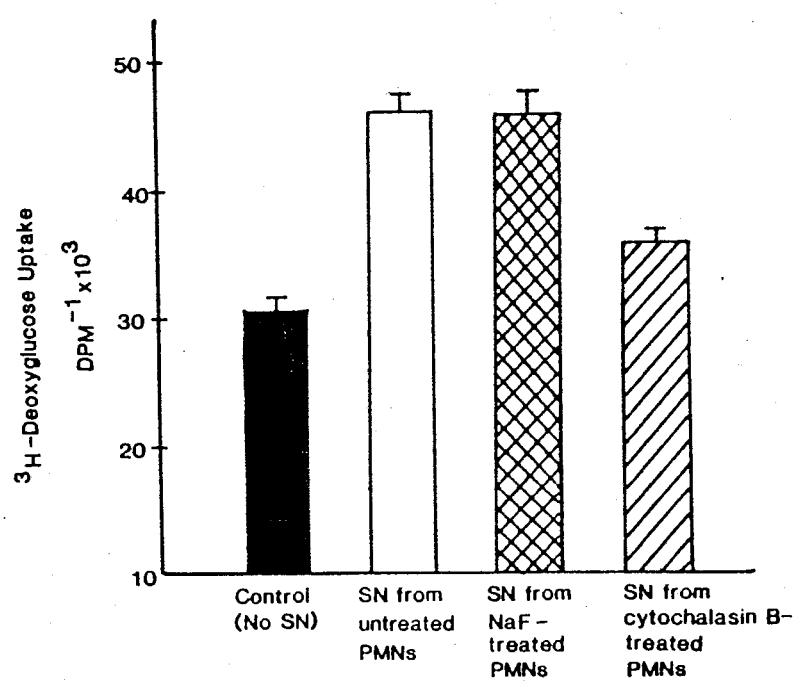


Figure 6.1 F. nucleatum-neutrophil interaction: effect of pre-treatment of neutrophils with sodium fluoride and cytochalasin B on the secretion of a soluble factor(s).

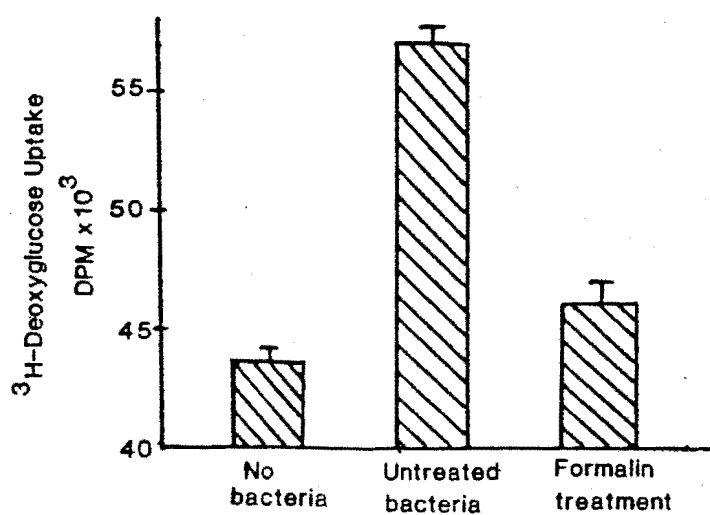
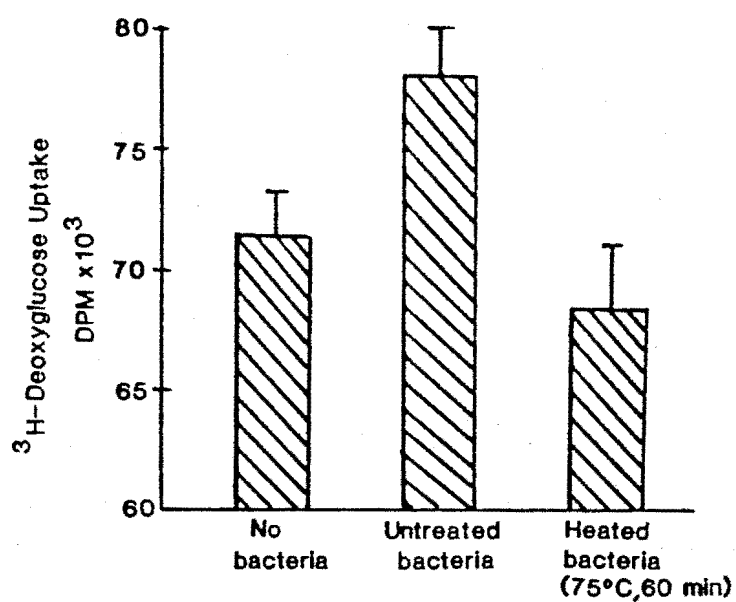


Figure 6.2 Effect of physiochemical treatment of *F. nucleatum* on secretion of a soluble factor(s) by neutrophils.

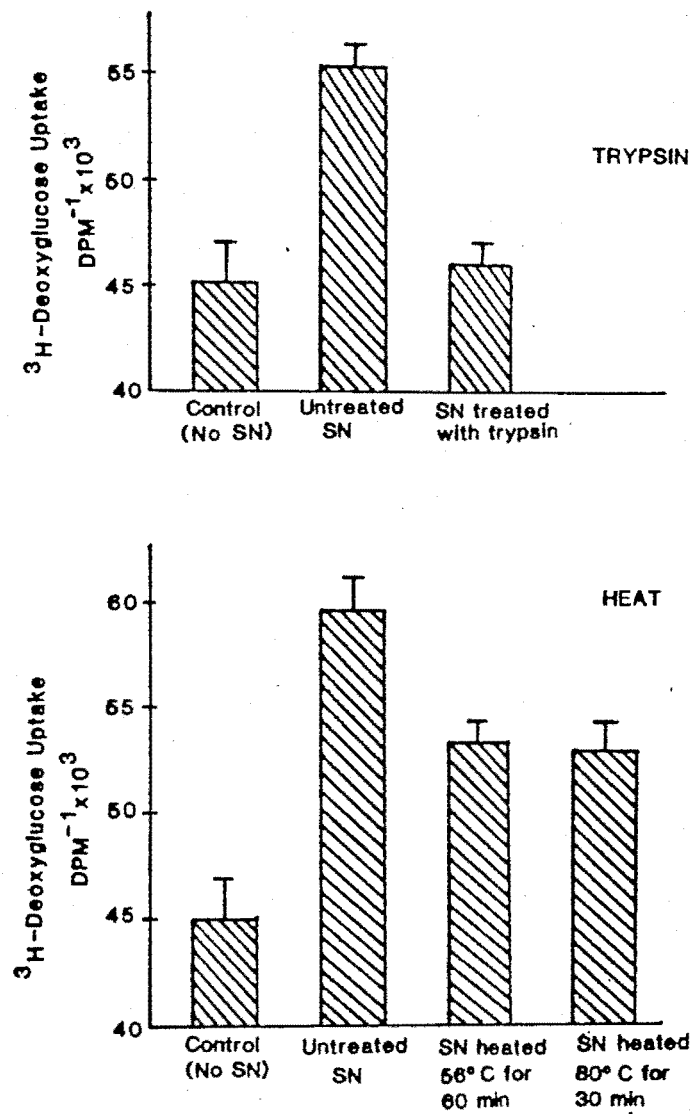


Figure 6.3 Effect of heat and trypsin treatment on the soluble factor obtained from *F. nucleatum*-neutrophil interactions.

reduced the activity of the soluble factor ($p < 0.05$).

Ultrafiltration studies

Finally, we passed the supernatant through Amicon Diaflo molecular filters (Amicon, NSW, Australia). We assayed the various fractions for ability to stimulate deoxyglucose uptake by neutrophils from a separate donor. The results (Table 6.3) show that activity was observed in fractions below 30,000 daltons, but not in fractions below 10,000 daltons. These results suggest that the soluble factor has a molecular weight of between 10,000 and 30,000 daltons.

Discussion

Neutrophils are phagocytic cells with vital roles in host defence and inflammation (Klebanoff, 1980; Weiseman et al, 1980). There is now increasing evidence to suggest that they have a critical role in immunoregulation as well (Vischer et al, 1976; Yamasaki & Ziff, 1977; Yoshinaga et al, 1980; Harris, 1982). Complex biological systems tend to have self-regulatory properties, and although this has been proposed for the neutrophil (Thong, 1982), self-regulatory factors have not been described until recently. While this manuscript was in preparation, Luciak et al (1986) reported the presence of a soluble factor after stimulation of

Table 6.3 Soluble factor from *F. nucleatum*-neutrophil interactions: ultrafiltration studies.

Ultrafiltration	³ H-deoxyglucose uptake DPM (mean ± S.D.)		
	30 mins	60 mins	90 mins
Control (medium)	28,676 ± 1,337	32,406 ± 2,640	37,476 ± 1,075
Unfiltered supernatant	40,462 ± 2,748*	46,137 ± 1,380*	60,591 ± 613**
30,000MW	35,503 ± 1,215*	44,648 ± 1,442*	59,424 ± 1,560**
10,000MW	29,917 ± 947	37,128 ± 2,150	41,868 ± 1,119
5,000MW	31,670 ± 1,034	34,392 ± 699	40,414 ± 1,139

Supernatant was obtained from *F. nucleatum*-neutrophil interaction for 60 mins. This was filtered through Amicon Diaflo YM 30, YM 10 and YM 5 ultrafilters, and the filtrate incubated with PMNs from a separate donor for 30, 60, and 90 mins and assayed for ³H-deoxyglucose uptake.

*p < 0.01

**p < 0.001

neutrophils by glass surfaces. This factor produced a dose-dependent decrease in Fc receptor expression of neutrophils. However, no further information was provided about the nature of this factor, or its approximate molecular weight.

We now report the discovery of a stimulus-specific self-regulatory factor capable of enhancing neutrophil adherence and deoxyglucose uptake. We chose to measure these modalities of neutrophil function because adherence is the earliest detectable change in neutrophil behaviour following stimulation, and perhaps one of the most crucial (Gallin, 1985), while deoxyglucose uptake is a quantitative measure of energy requirement for neutrophil adherence, chemotaxis, phagocytosis, and other vital cell functions as well as the generation of oxygen radicals (Seow et al, 1987e; McCormack et al, 1981; Borregaard & Herlin, 1982; Babior, 1978). This enhancing factor was secreted on stimulation with *F. nucleatum* but not *B. gingivalis*. In fact, interaction of *B. gingivalis* with neutrophils resulted in the secretion of a soluble factor with inhibitory effects on neutrophil adherence. It would appear that there may be a number of neutrophil self-regulatory factors in existence, and from current knowledge about interleukins and other cytokines, there may be only a small number of them despite the large numbers of potential stimuli.

The biological role of these soluble factors may be to act as molecular mediators for the control or amplification of neutrophil and inflammatory responses. Thus it is expected that some of these factors would be down-regulatory (suppressive) and others up-regulatory (enhancing).

The *F. nucleatum*-induced enhancing factor reported here has been partially characterised. It is secreted within 15 mins. of *F. nucleatum*-neutrophil interaction. Secretion of the factor was inhibited by pre-treatment of neutrophils with cytochalasin B but not sodium fluoride. Alteration of the bacterial cell surface by heat or formalin markedly reduced factor secretion. The molecular weight was found to be between 10,000 and 30,000 daltons. The enhancing factor was sensitive to heat and trypsin. This indicates that it is probably a protein. It also excludes the possibility of it being endotoxin, because endotoxins are heat resistant (Hibbs et al, 1977).

This discovery of a stimulus-specific neutrophil self-regulatory factory may have important implications in the pathogenesis and treatment of periodontitis. Whereas the release of a suppressive factor after *B. gingivalis*-neutrophil interaction would aid the colonisation of the periodontal pocket by this bacteria, and

indeed *B. gingivalis* has been shown to be very successful in this regard, (Tanner et al, 1979; White & Maynard, 1981; Slots, 1977) the secretion of an enhancing factor after *F. nucleatum*-neutrophil interaction would result in the release of toxic oxygen radicals and lysosomal enzymes by neutrophils, thus giving rise to inflammatory changes and periodontal tissue destruction seen in both initial and established periodontal disease (Page & Schroeder, 1976; Weissman et al, 1980; Miller et al, 1984).

It would seem worthwhile to fully characterise and clone some of these bacteria-specific neutrophil self-regulatory factors as a means of understanding the cell biology of neutrophils in molecular terms. Also, bacterial diseases continue to account for enormous worldwide morbidity and mortality despite the availability of potent antimicrobial agents; better knowledge about bacteria-neutrophil interactions and molecular mechanisms of pathogenesis may lead to novel strategies for treatment and prevention.

CHAPTER SEVEN

AUGMENTATION OF HUMAN POLYMORPHONUCLEAR NEUTROPHIL
ADHERENCE BY INTERFERON

Introduction

The interferon system consists of a family of proteins with antiviral, antitumour and immunomodulatory activities. These proteins are classified into three broad groups. Alpha-interferon, of which there are at least ten subtypes, is produced by leukocytes stimulated with virus or polyribonucleotides. Beta-interferon is formed by fibroblast cultures on stimulation with the same agents. Gamma-interferon is produced by T-lymphocytes in response to antigens and mitogens (Steward, 1979; Johnson, 1983; Friedman & Vogel, 1983; Woodrow, 1983; De Maeyer & De Maeyer-Guinard, 1982).

That the interferons have wide-ranging effects on both the humoral and cell-mediated components of the immune system is well documented (Woodrow, 1983; Johnson, 1983; Friedman & Vogel, 1983). However, relatively few reports are available on the modulation

of polymorphonuclear neutrophil (PMN) function. Enhancement by interferon of in vitro neutrophil phagocytosis (Melby et al, 1982; Jarstrand & Einhorn, 1983), hexose-monophosphate (HMP) shunt activity (Ferrante & Rencis, 1984), and antibody-dependent cell-mediated cytotoxicity (ADCC) (Hokland & Berg, 1981) have been previously observed. Augmentation of nitroblue tetrazolium (NBT) dye-reduction by interferon has also been observed in vitro (Jarstrand & Einhorn, 1983) and in vivo (Einhorn & Jarstrand, 1984). The above aspects of neutrophil function occur later in the sequence of events following neutrophil activation, whereas adherence occurs earlier and precedes the migration of neutrophils to sites of inflammation and infection. Modulation of neutrophil adherence has been shown with biologically active molecules such as prostaglandins (Boxer et al, 1980), leukotrienes (Palmlblad et al, 1981), and activated complement components (McGillen et al, 1980). In this study we examined the effects of interferon on this early and crucial event of neutrophil function.

Materials and Methods

Interferons

The interferons used in the experiments were obtained from Sigma Chemical Company (St. Louis, Mo).

Highly purified human lymphoblastoid interferon (IFN- α) with an activity of 2×10^8 IU/mg protein (Cat.No. I-7257) was produced in Burkitt lymphoma cells by induction with Sendai virus. This subtype of IFN- α was used throughout the experiments. Human leukocyte interferon (IFN- α) with an activity of 4×10^6 IU/mg protein (Cat.No. I-1008) was produced in leukocytes primed with leukocyte interferon followed by Sendai virus. This subtype of IFN- α was used in the last sets of experiments. Human gamma interferon (IFN- γ) with an activity of 1×10^6 IU/mg protein (Cat.No. I-6507) was produced from human buffy coats by induction with A23187 and mezerin.

Neutrophil isolation

Heparinised blood was obtained from healthy donors by venepuncture. About 7ml of blood was layered on 3ml of Mono-Poly Resolving Medium (Flow Laboratories, Va), and centrifuged at 1000g for 40 mins. (Ferrante & Thong, 1980). This one-step procedure resulted in two bands at the interface. Neutrophils were removed from the second band, washed and resuspended in medium 199, and adjusted to a concentration of $8-10 \times 10^6$ per ml. They were of >97% purity.

Neutrophil adherence microassay

The microassay for neutrophil adherence was performed as previously described (Thong & Currell, 1983). Nylon fibre microcolumns were prepared by carefully weighing out 10mg lots of teased nylon fibres (Olympic Products, Queensland), and evenly packed into 100 μ l disposable pipette tips (Stockwell Scientific, Ca), so as to occupy the central 2cm of the 5cm length. Each microcolumn so prepared can easily accommodate 0.1ml of the neutrophil suspension.

Equal volumes of neutrophil suspension and interferon solutions were mixed and incubated at 37°C for varying periods (incubation time) before delivery into the nylon microcolumns. The nylon fibre microcolumns were then placed in an incubator at 37°C and high humidity for varying time periods (contact time), after which they were placed in a specially designed apparatus (Thong & Currell, 1983). The fluid was extracted into disposable test tubes by a vacuum suction pressure of 250 millibars applied for 1 min. The concentration of neutrophils was determined by Neubauer haemocytometer and the results calculated as follows:

$$\text{Percent adherence} = 100 - \frac{\text{CE}}{\text{CA}} \times 100$$

CA where CE is concentration of neutrophils in effluent and CA is concentration of neutrophils in original suspension.

These experiments were performed in triplicate and results expressed as mean \pm SE. The student's t-test was used for statistical analysis.

Results

Dose-response effects of IFN on neutrophil adherence

Preliminary experiments indicated that incubation times of 5 mins. followed by contact times of 5 mins. are suitable for use in these experiments. The results of various concentrations of IFN- α and IFN- γ are shown in Figure 7.1. It can be seen that the optimal concentrations for these interferons lie between 100 to 1000 IU/ml. For IFN- α percent neutrophil adherence increased from a baseline of 58.6 ± 6.1 to 78.2 ± 8.9 at 1000 IU per ml. ($p < 0.05$). For IFN- γ , percent neutrophil adherence increased from a baseline of 67.1 ± 3.2 to 88.8 ± 1.3 ($p < 0.001$) at 1000 IU/ml. At the high concentrations of 10,000 IU/ml, neutrophil adherence values were generally lower than optimal. This dose-response relationship conforms with recognised physiological behaviour of neutrophils (Palmlblad et al, 1981; Neidel et al, 1979).

Effects of varying incubation times with IFN on neutrophil adherence

The time course of IFN and neutrophil interaction

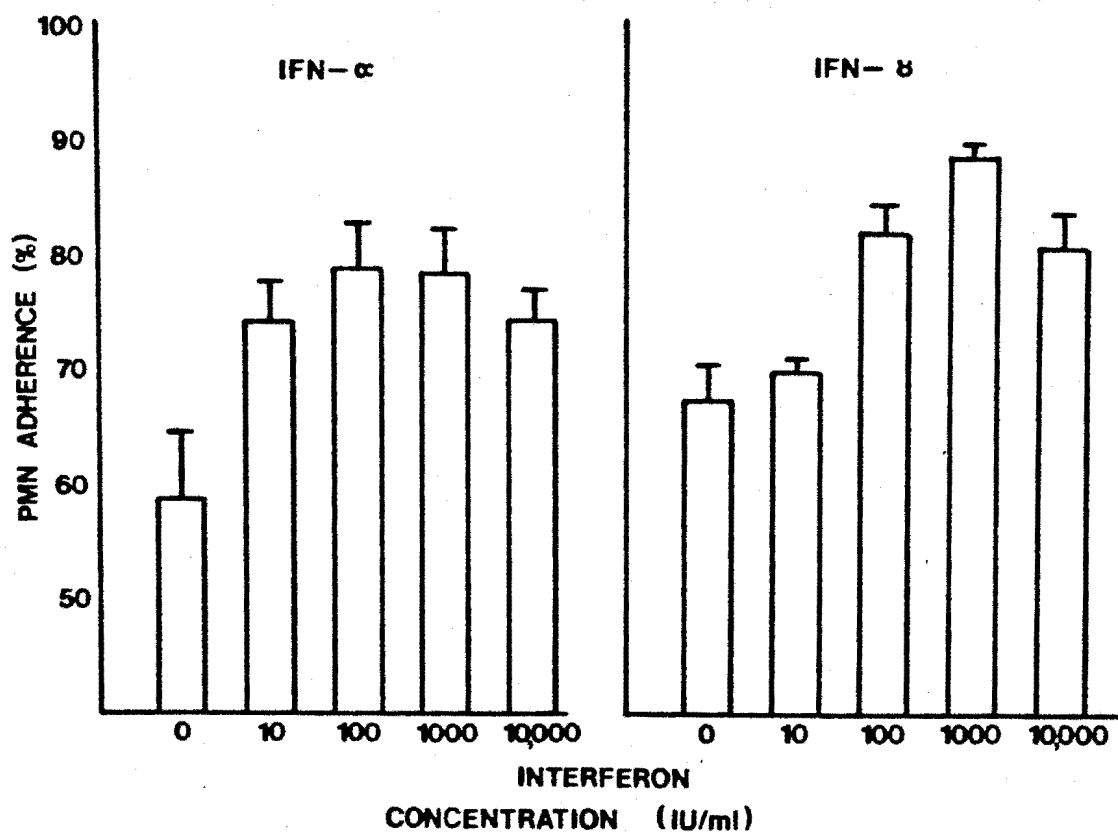


Figure 7.1 Effect of varying concentrations of IFN-α and IFN-γ on PMN adherence. Incubation time = 5 mins, contact time = 5 mins.

was investigated in this set of experiments. The IFN concentration of 100 IU/ml was selected for both IFN- α and IFN- γ , and contact times were kept at 5 mins. The results (Figure 7.2) show that enhancement of neutrophil adherence occurred as early as 2 mins. after incubation with IFN. For IFN- α , percent neutrophil adherence rose from a baseline of 61.9 ± 3.0 to 85.6 ± 3.2 at 2 mins. ($p < 0.001$), and subsequently fell to 46.3 ± 3.5 at 60 mins ($p < 0.01$). For IFN- γ , percent neutrophil adherence increased from a baseline of 62.3 ± 2.0 to 67.6 ± 1.4 at 2 mins. ($p < 0.05$), and 73.9 ± 0.86 at 5 mins ($p < 0.001$), but decreased to 64.2 ± 3.9 at 60 mins ($p > 0.1$). These time-response relationships are reminiscent of deactivation phenomenon seen in other neutrophil responses (Boxer et al, 1980; Zigmond, 1977).

Effects of IFN on the kinetics of neutrophil adherence

The extent of neutrophil adherence is directly proportional to the time allowed for contact between neutrophils and nylon fibre microcolumns (Thong & Currell, 1983; Kelly & Thong, 1984).

Although previous experiments were performed with contact times of 5 mins, this can be varied because of the unique design of our microcolumns which retained the neutrophil suspensions by means of capillary forces until extraction of the fluid by the vacuum harvester at

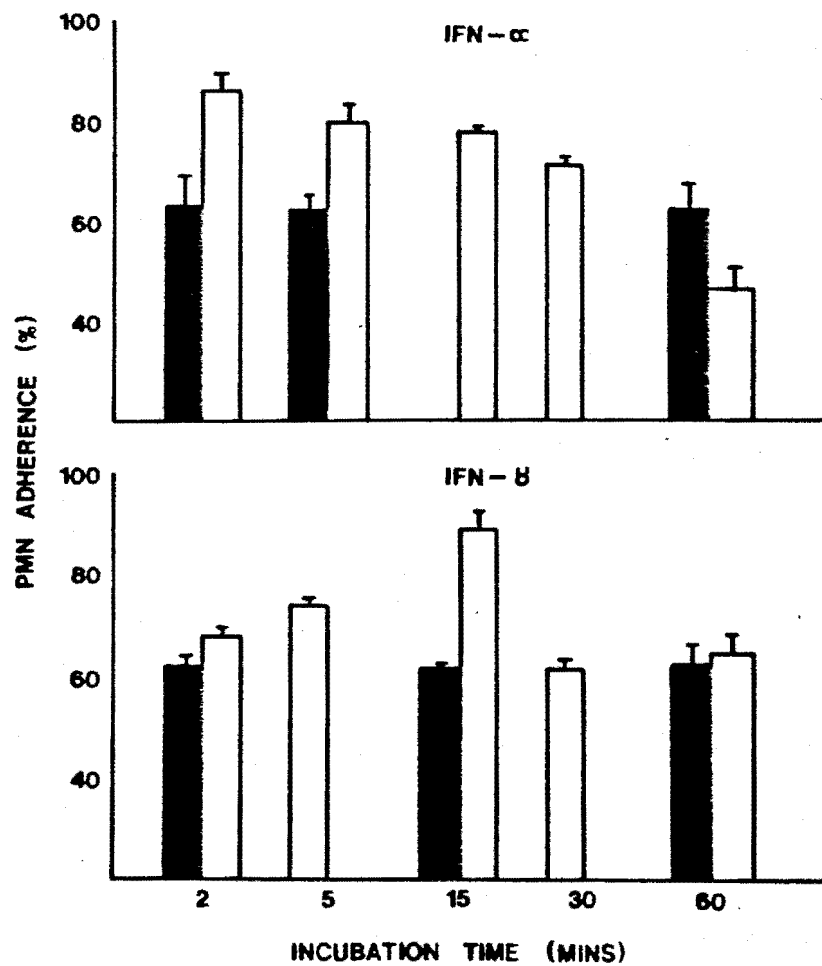


Figure 7.2 Effect of varying incubation times with IFN- α and IFN- γ on PMN adherence. IFN concentration = 100 IU/ml, contact time = 5 mins.

a precise time.

It can be seen that both IFN- α and IFN- γ enhanced neutrophil adherence at all contact times (Figure 7.3). At a concentration of 100 IU/ml, IFN- α enhanced neutrophil adherence from 21.4 ± 6.2 percent to 77.0 ± 5.1 percent at a contact time of 3 mins. ($p < 0.001$), and similar trends were seen for subsequent contact time periods, of 6, 9 and 12 mins.

At a similar concentration, IFN- γ enhanced neutrophil adherence from 35.0 ± 1.2 percent to 56.0 ± 0.7 percent at a contact time of 3 mins. ($p < 0.001$), and similar trends were seen for subsequent time periods.

Synergistic effect of IFN- α and IFN- γ on neutrophil adherence

The effects of IFN- α and IFN- γ are mediated through different membrane receptors (Branca & Baglioni, 1981), and synergism between these two interferons have been shown to occur in lymphocyte natural killer (NK) cell activity (Weigent et al, 1983).

Synergism between IFN- α and IFN- γ on neutrophil adherence can also be deduced from our data (Figure 7.4). Using low concentrations of IFN, we found that incubation of neutrophils with 8 IU/ml of IFN- γ produced

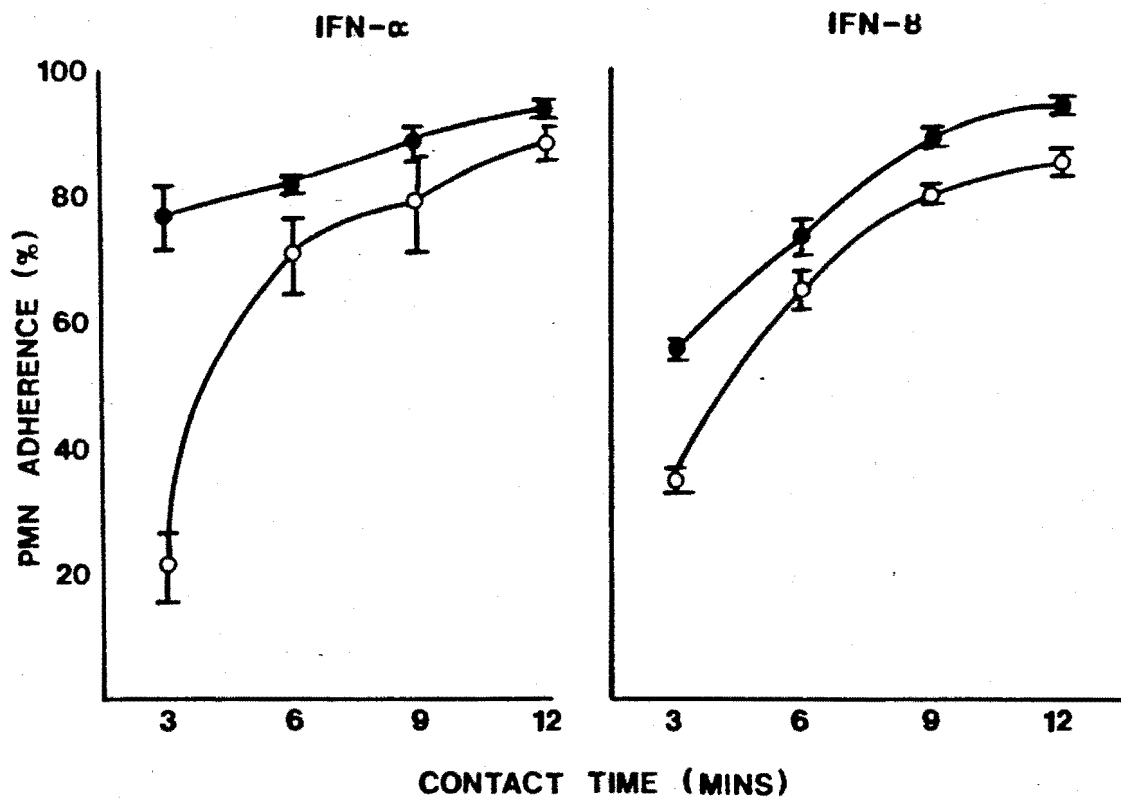


Figure 7.3 Effect of IFN- α and IFN- γ on the kinetics of PMN adherence. PMNs from the same donor was incubated with either 100 IU/ml of IFN for 5 mins (closed circles), or without IFN (open circles), and adherence measured at different contact times. Separate donors were used for experiments with IFN- α and IFN- γ .

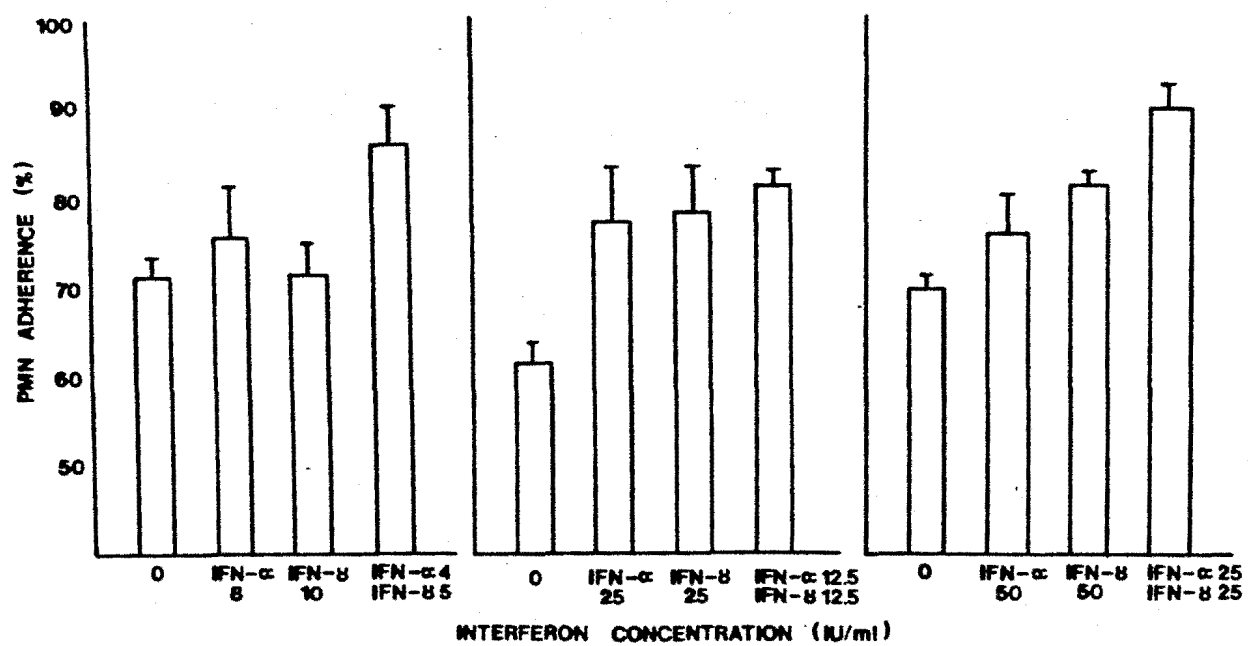


Figure 7.4 Synergistic effects of IFN- α and IFN- γ on PMN adherence. Incubation time = 5 mins, contact time = 5 mins.

an adherence value of 76.0 ± 4.2 percent ($p > 0.1$), a slight but insignificant increase over the baseline value of 72.0 ± 1.8 percent. Similarly, 10 IU/ml of IFN- γ gave a value of 72.4 ± 2.9 percent ($p > 0.1$). However, a combination of only 4 IU/ml of IFN- α and 5 IU/ml of IFN- γ enhanced neutrophil adherence to 85.8 ± 3.1 percent ($p < 0.05$).

The combinations of these two IFNs at a concentration of 12.5 IU/ml each raised neutrophil adherence from 61.0 ± 1.7 percent to 81.2 ± 1.1 percent ($p < 0.001$), well above the individual values of IFN- α (25 IU/ml) or IFN- γ (25 IU/ml) on their own.

At high concentrations of IFN, we found that IFN- α (50 IU/ml) raised neutrophil adherence from 69.0 ± 1.1 percent to 76.1 ± 3.2 percent ($p < 0.05$) whereas IFN- γ at the same concentration elevated neutrophil adherence to 81.6 ± 1.1 percent ($p < 0.01$). However, the combination of these two IFNs at half the previous concentrations (25 IU/ml + 25 IU/ml) raised it to the higher value of 88.6 ± 1.9 percent ($p < 0.001$).

Non-synergism between two subtypes of IFN- α

Since IFN- α subtypes share the same receptor (Branca & Baglioni, 1981), it is not expected that the combination of two subtypes of IFN- α would produce

synergism. This is in fact the case (Table 7.1). At low concentrations of the two subtypes, each on its own raised adherence but not to a statistically significant degree; in combination, no synergism was observed. Their combination at the higher dose of 12.5 IU/ml increased adherence from 58.3 ± 1.6 percent to 65.9 ± 3.2 percent only, a value lower than that given by each IFN- α subtype on its own. A similar trend was observed with a combination of 25 IU/ml of both subtypes of IFN- α .

Effect of heat treatment on IFN

Interferons are heat-sensitive, and depression or abolition of activity has been shown by heat-treatment at between 60°C and 80°C for 30 mins (Pak et al, 1980). We treated the three types of interferon preparations used in our experiments for 30 mins at 65°C, and the results are presented in Table 7.2. It can be seen that for each type of interferon, neutrophil adherence was significantly increased after incubation with unheated IFN. In contrast, heat-treated IFNs gave similar values to controls.

Discussion

The phenomenon of neutrophil adherence was first observed in the mesentery of the frog by Cohnheim in 1882. It is the earliest detectable change in neutro-

Table 7.1 Non-synergism between two subtypes of alpha interferon

	Control	IFN- α	IFN- $\underline{\alpha}$	IFN- α + IFN- $\underline{\alpha}$
Exp. 1				
IFN conc. (IU/ml)	0	8	10	4 + 5
% Adherence (Mean \pm S.E.)	42.2 \pm 4.2	52.3 \pm 1.8	49.3 \pm 1.4	48.3 \pm 1.4
Exp. 2				
IFN conc. (IU/ml)	0	25	25	12.5 + 12.5
% Adherence (Mean \pm S.E.)	58.3 \pm 2.2	71.5 \pm 2.0*	69.8 \pm 2.4**	65.9 \pm 4.9
Exp. 3				
IFN conc. (IU/ml)	0	50	50	25 + 25
% Adherence (Mean \pm S.E.)	42.2 \pm 3.8	58.0 \pm 5.4†	57.5 \pm 4.2†	49.6 \pm 5.3

Each experiment was performed with PMNs obtained from a different donor.

* $p < 0.01$

** $p < 0.02$

† $p < 0.05$

Table 7.2 Effect of heat treatment on interferon augmentation of PMN adherence

Experiment	IFN Type (1,000 IU/ml)	% Adherence \pm S.E.		
		Control	IFN Unheated	IFN Heated (65°C/30 mins)
1	IFN- α	56.2 \pm 0.4	68.0 \pm 2.2*	51.8 \pm 0.7
2	IFN- α	42.1 \pm 3.1	52.2 \pm 1.5**	37.1 \pm 1.1
3	IFN- γ	40.3 \pm 0.8	55.9 \pm 1.4†	37.9 \pm 3.8

Each experiment was performed with PMNs obtained from a different donor.

* $p < 0.01$

** $p < 0.05$

† $p < 0.001$

phil behaviour, and perhaps one of the most crucial. Neutrophil adherence is mediated by a surface glycoprotein made up of two peptide chains of 155 and 94 kilodaltons (Dana et al, 1984). The congenital absence of this glycoprotein results in increased susceptibility to infection (Crowley et al, 1980). Modulation of neutrophil adherence by inflammatory molecules is well documented. Thus, activated complement components (McGillien et al, 1980), chemotactic peptides (Fehr & Dahinden, 1979) and leukotriene B₄ (McGillien et al, 1980) stimulate adherence, while prostacyclin (Boxer et al, 1980; McGillien et al, 1980) inhibits adherence. Since IFN can be considered an inflammatory molecule produced in response to viruses and other inducers, it would be pertinent to examine its effects on neutrophil adherence.

The results of the present studies indicate that IFN augments neutrophil adherence. Optimum effects were observed at concentrations of 100-1000 IU/ml. Adherence was lower than optimal at higher concentrations. Similar dose-response relationships have been observed with neutrophil adherence response to leukotriene B₄ (Palmlblad et al, 1981) and neutrophil chemotactic responses to formyl peptides (Neidel et al, 1979). The response to IFN was very rapid; enhancement of adherence was evident by 2 mins. and was sustained for up

to 30 mins. before subsiding. Similarly, rapid neutrophil adherence responses have been observed after incubation with chemotactic peptide (Boxer et al, 1979), prostacyclin (Boxer et al, 1980), and leukotriene B₄ (Palmlblad et al, 1981). The decay of neutrophil adherence after prolonged incubation with IFN probably represents deactivation phenomenon, and has been reported with prostacyclin (Boxer et al, 1980); it has also been observed with neutrophil orientation or chemiluminescence in response to chemotactic peptides (Zigmond, 1977; Jadwin et al, 1981; Dahlgren et al, 1982), or complement components during phagocytosis (Wright & Gallin, 1975). Our data further demonstrate that IFN- α and IFN- γ have synergistic effects on neutrophil adherence. This has been previously shown for lymphocyte NK activity (Weigent et al, 1983), and conforms to data from molecular studies that each type of IFN binds to different receptors (Branca & Baglioni, 1981).

Neutrophils are phagocytic cells with vital roles in host resistance and inflammation (Klebanoff, 1980; Weissman et al, 1980). They are among the first cells to congregate at sites of infection, arriving within 30 mins. as shown by radio-labelling (Dutcher et al, 1981). It has also been shown that their arrival within the first 2-4 hours is critical for the control of infection

(Miles et al, 1957). It is therefore not surprising that there is interaction between IFN and neutrophils, since IFN is produced very early in the course of viral infections (Stanton et al, 1978), and a viricidal effect has been demonstrated for neutrophils (Klebanoff, 1980; Seibens et al, 1979). Previous studies have shown enhancement of a broad range of neutrophil functions by interferon. Melby et al (1982) reported that human neutrophils ingested more radio-labelled bacteria after 3 hours incubation with an optimal concentration of 1,000 IU/ml of IFN- α . Jarstrand and Einhorn (1983) reported that human neutrophils had increased phagocytosis and NBT dye reduction but not chemotaxis when exposed to 10-1,000 IU/ml of both IFN- α and IFN- β for 30 mins. Ferrante and Rencis (1984) demonstrated that the HMP shunt activity in human neutrophils was stimulated by incubation with 5,000 IU/ml of IFN- β for 90 mins. Hokland and Berg (1981) found that incubation of human neutrophils with 100-1,000 IU/ml of IFN- α for 30-60 minutes resulted in enhancement of ADCC activity against erythrocyte and tumour target cells. Pak et al (1980) investigated the effects of 125 to 4,000 IU/ml of IFN- α and IFN- β on human neutrophils and found significant enhancement of NBT dye reduction.

One unconfirmed report (Farr et al, 1983) indicated lack of activity of recombinant IFN- α_2 on several aspects

of neutrophil function. These investigators suggested that their discrepant results may be due to the particular type of IFN they were using, or because previous workers have used contaminated IFN. Bacterial endotoxin is the most common contaminant of biological preparations (Hibbs et al, 1977), but it is quite unlikely that other IFN preparations are contaminated with endotoxin or other contaminants for several reasons. First, some of the previous studies (Jarstrand & Einhorn, 1983; Hokland & Berg, 1981; Pak et al, 1980), including our own, have used highly purified IFNs with specific activities equal to that of 1×10^8 IU/mg protein used by Farr et al (1983). Second, all previous studies (Melby et al, 1982; Jarstrand & Einhorn, 1983; Ferrante & Rencis, 1984; Hokland & Berg, 1981; Pak et al, 1980) have used mock interferon as control for their experiments. Third, anti-IFN anti-sera have been employed (Melby et al, 1982; Hokland & Berg, 1981; Pak et al, 1980) as addition controls. Fourth, whereas endotoxins are heat resistant (Hibbs et al, 1977), the heat sensitivity of IFN preparation has been demonstrated by Pak et al (1980) and ourselves. Fifth, it is unlikely that the synergistic effects between IFN- α and IFN- γ but not between two subtypes of IFN- α , as shown in our experiments, be due to endotoxin. Thus, the discrepant findings of Farr et al (1983) may be best explained on the basis of a difference between the recombinant IFN

they were using and the leukocyte and fibroblast IFNs used by other workers, which consist of a mixture of IFN types and subtypes, some of which may have modulatory effects on neutrophil function, and others not.

In conclusion, most studies indicate that interferon may have stimulatory effects on a large spectrum of neutrophil function (Melby et al, 1982; Jarstrand & Einhorn, 1983; Ferrante & Rencis, 1984; Hokland & Berg, 1981; Pak et al, 1980; Einhorn & Jarstrand, 1984), in addition to its modulatory effects on humoral and cell-mediated immunity (Johnson, 1983; Friedman & Vogel, 1983; Woodrow, 1983; De Maeyer & De Maeyer-Guinard, 1982; Onsrud, 1982). The biologic role of the interferon system may include augmentation of neutrophil activity in the control of microbial (Klebanoff, 1980; Seibens et al, 1979) or tumour invasion (Gale & Zigelboim, 1975; Katz et al, 1980) in non-immune individuals, or in the early stages of invasion when specific immunological mechanisms are not yet fully mobilised.

CHAPTER EIGHT

LYMPHOCYTE-NEUTROPHIL INTERACTIONS: OPPOSITE EFFECTS OF
INTERLEUKIN-2 AND TUMOUR NECROSIS FACTOR-BETA
(LYMPHOTOXIN) ON HUMAN NEUTROPHIL ADHERENCE

Introduction

Neutrophil leukocytes have a primary role as effector cells in the killing of microbial pathogens, but the participation of cell-mediated immunological processes is required for the optimal performance of neutrophils in many infectious diseases, eg, fungal and mycobacterial infections (Mason & Kirkpatrick, 1983). Activated T lymphocytes secrete a number of lymphokines which serve as molecular mediators for the recruitment of other leukocytes in order to amplify immunological and inflammatory responses (Smith, 1984; Dinarello & Meir, 1986; Gray et al, 1984), and these lymphokines may be involved with modulation of neutrophil behaviour. In this regard, it has been previously demonstrated that polyclonal activation of human lymphocytes by T and B cell mitogens results in the release in culture fluid of cytokines which stimulate the capacity of human neutro-

phils to kill pathogenic amoebae, bacteria, fungi and tumour cells, and enhance those biochemical components of the neutrophils which are involved with the microbicidal process (Ferrante & Mocatta, 1984; Ferrante & Abell, 1986; Ferrante et al, 1987). It has also been demonstrated that purified or recombinant cytokines such as granulocyte-macrophage colony stimulating factors, interferons and cachectin, modulate neutrophil functions (Vadas et al, 1984; Ferrante & Rencis, 1984; Weisbart et al, 1985; Seow & Thong, 1986a; Weisbart et al, 1986). However, the importance of T lymphocyte-neutrophil interactions in the expression of immunity to infection has not been completely defined. In this report, we examined the effects of two well-characterised lymphokines, interleukin-2 (IL-2) and tumour necrosis factor-beta ($\text{TNF-}\beta$) on neutrophil adherence, which represents one of the earliest steps in the recruitment of neutrophils at infections sites (Gallin, 1985). The results demonstrate that IL-2 and $\text{TNF-}\beta$ are neutrophil modulators.

Materials and Methods

Lymphokines

Recombinant human IL-2 (ala-125), obtained from Amersham International, UK, is designated Batch No. 10 and has a specific activity of 2.8×10^3 units/ml.

Recombinant (E.coli derived) human TNF- β was kindly provided by Dr G. R. Adolf, Ernst-Boehringer Institut, Vienna, Austria, and has specific activity of 1.2×10^8 units/mg when assayed for cytotoxicity on mouse L-M cells.

Neutrophil isolation

Approximately 20ml of heparinised blood was obtained from healthy donors by venepuncture, layered onto Mono-Poly Resolving Medium (Flow Laboratories, Va), (Ferrante & Thong, 1980; 1982), and centrifuged at 400G for 30 min. This one-step technique resulted in the formation of two leukocyte bands at the interface. Neutrophils were removed from the second band, washed twice, resuspended in medium 199, and adjusted to a concentration of $8-10 \times 10^6$ cells/ml. They were of >97% purity (Ferrante & Thong, 1980; 1982).

Neutrophil adherence

This microassay was performed as previously described (Thong & Currell, 1983). Briefly, microcolumns were prepared by carefully weighing out 10mg lots of dacron fibre (Olympic General Products, Qld), and evenly packing them into 100 μ l disposable pipette tips (Stockwell Scientific, Ca), so as to occupy the middle 2 cm of the 5 cm length. Each microcolumn so prepared can easily accommodate 0.1ml of the neutrophil

suspension.

Equal volumes of neutrophil suspension and lymphokine solution were mixed and incubated at 38°C for varying periods of time (incubation time) before delivery into the microcolumns. The microcolumns were then placed into an incubator at 37°C in humidified air for 5 min. The fluid was extracted into disposable test tubes by a vacuum pump operating a suction pressure of 250 mbar applied for 2 min. The concentration of neutrophils was determined by Neubauer haemocytometer, and the results calculated as follows:

$$\text{Percentage adherence} = 100 - \frac{\text{CE}}{\text{CA}} \times 100$$

where CA is the concentration of neutrophils applied to the microcolumn, and CE the concentration recovered in the effluent.

Experiments were performed in triplicate and results expressed as mean \pm SD. The student's t-test was used for statistical analysis.

Results

Dose-response

Neutrophils were incubated with various concentrations of either IL-2 or TNF- β for 15 min. prior to experiments. The results are presented in Figure 8.1. It can be observed that IL-2 at concentrations of 20 and 200 units/ml caused significant depression of neutrophil adherence to 29.4 ± 5.1 (% mean \pm SD) and 28.8 ± 2.8 respectively ($p < 0.01$). At 2 units/ml, IL-2 gave similar values to control: 54.8 ± 2.3 vs. 55.8 ± 1.9 .

By contrast, TNF- β at 500 units/ml caused significant enhancement of neutrophil adherence to 67.3 ± 3.4 from a baseline of 43.3 ± 6.3 ($p < 0.001$). At 20 and 100 units/ml, TNF- β did not significantly increase neutrophil adherence.

Time course

We next determined the effects of varying incubation times with IL-2 (200 units/ml) and TNF- β (500 units/ml). The results (Figure 8.2) showed no detectable change in neutrophil adherence after 5 min. incubation. However, by 15 min. significant depression by IL-2 and enhancement by TNF- β of neutrophil adherence was readily observed, and these opposite effects of the two lymphokines were sustained for at least 60 min.

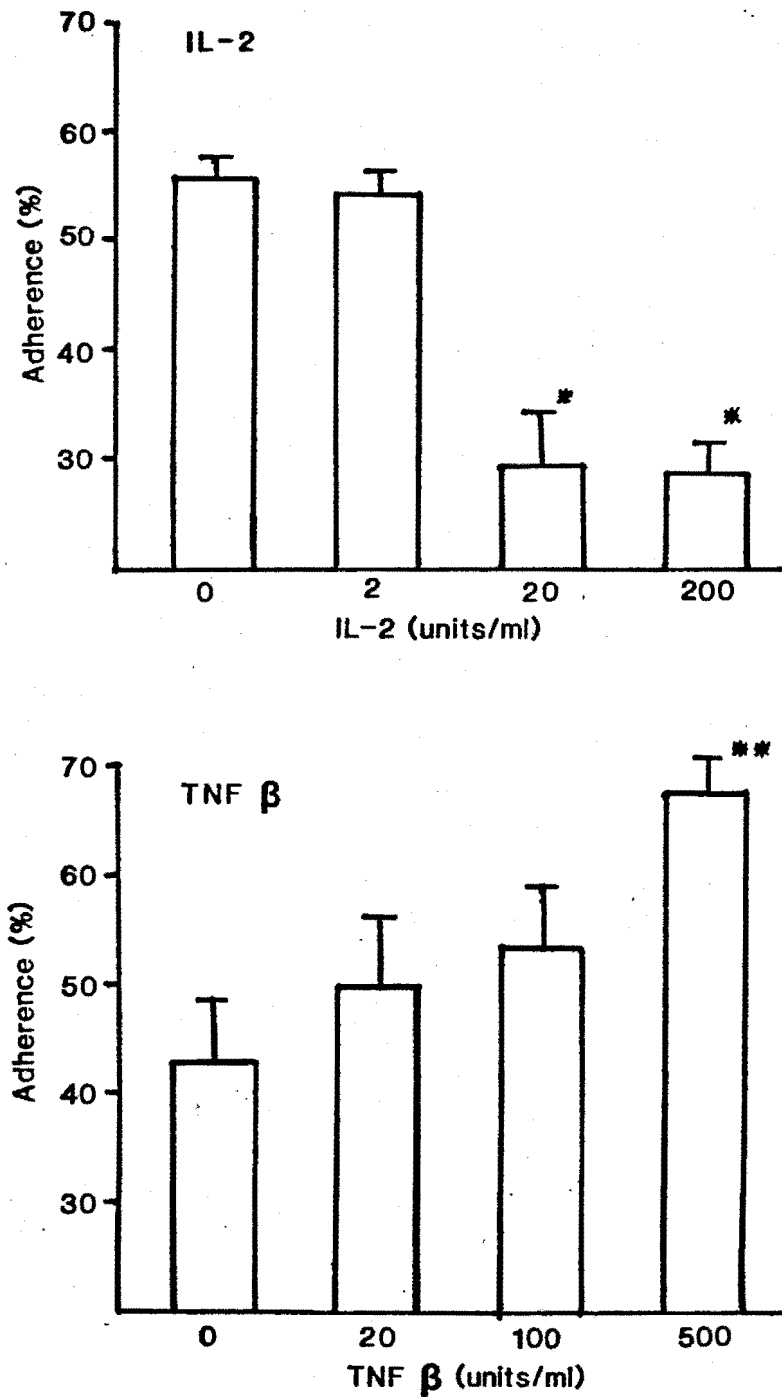


Figure 8.1 Effect of varying concentrations of IL-2 and TNF β on human neutrophil adherence. Various concentrations of lymphokine were incubated with neutrophils for 30 min.

* $p < 0.01$
** $p < 0.001$

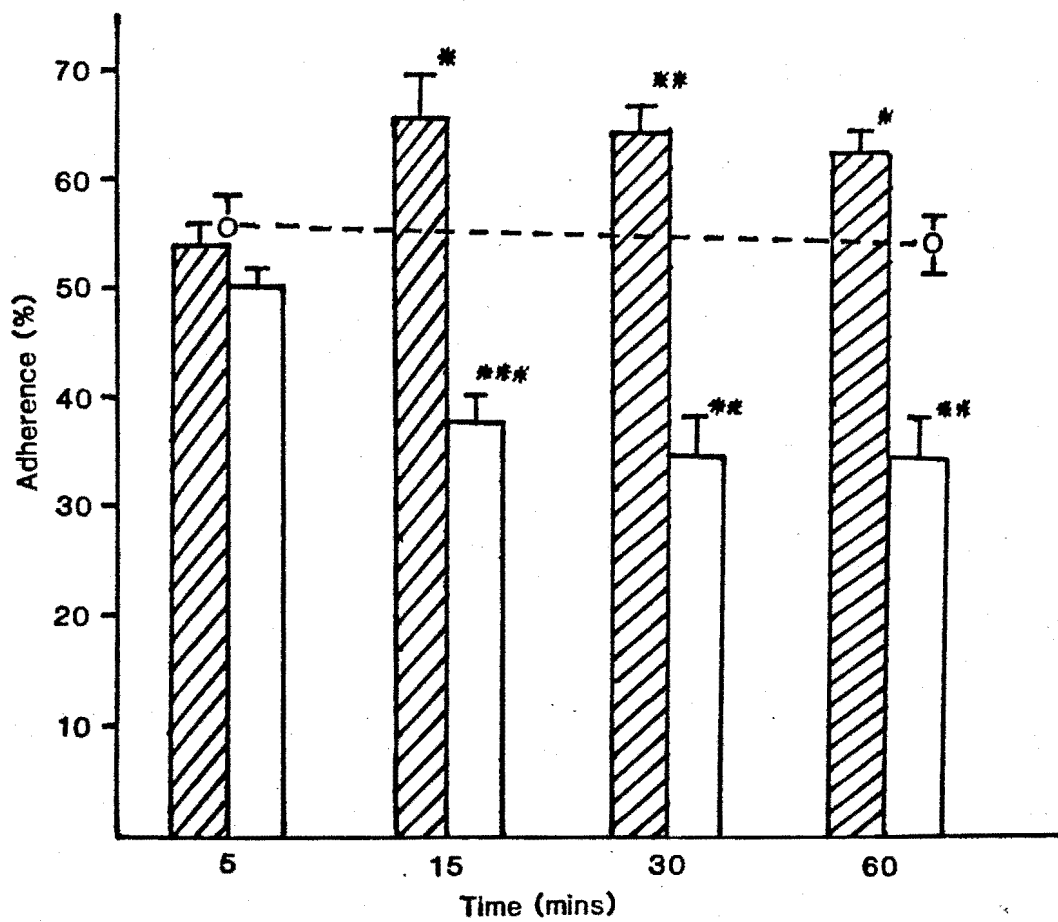


Figure 8.2 Time course of IL-2 (\square) and TNF β (hatched) effects on human neutrophil adherence (\circ Control)

* $p < 0.02$
 ** $p < 0.01$
 *** $p < 0.001$

Combined effects

The combined effects of IL-2 and TNF- β were examined in the next set of experiments (Figure 8.3). Again, it can be seen that IL-2 (200 units/ml) and TNF- β (500 units/ml) caused significant depression and enhancement of neutrophil adherence, respectively. In combination, the opposite effect of these two lymphocytes cancelled out each other so that neutrophil adherence was not significantly different from control.

Effect of FMLP

Formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma Chemical Company, St. Louis) is a synthetic bacterial peptide with stimulatory effects on a number of neutrophil functions (Williams & Cole, 1981; Dahlgren et al, 1982). At a concentration of 4×10^{-7} M, FMLP significantly increased human neutrophil adherence (Table 8.1). Again, IL-2 suppressed neutrophil adherence from 48.2 ± 2.8 percent to 23.4 ± 1.5 percent ($p < 0.01$); this lymphokine was able to counteract the stimulation achieved by FMLP from 65.1 ± 2.3 percent to 46.0 ± 2.1 percent ($p < 0.01$).

TNF- β again enhanced neutrophil adherence from 48.2 ± 2.8 percent to 63.2 ± 0.8 percent ($p < 0.001$). The combination of TNF- β and FMLP was able to enhance adherence to 75.7 ± 2.5 percent ($p < 0.01$), greater than

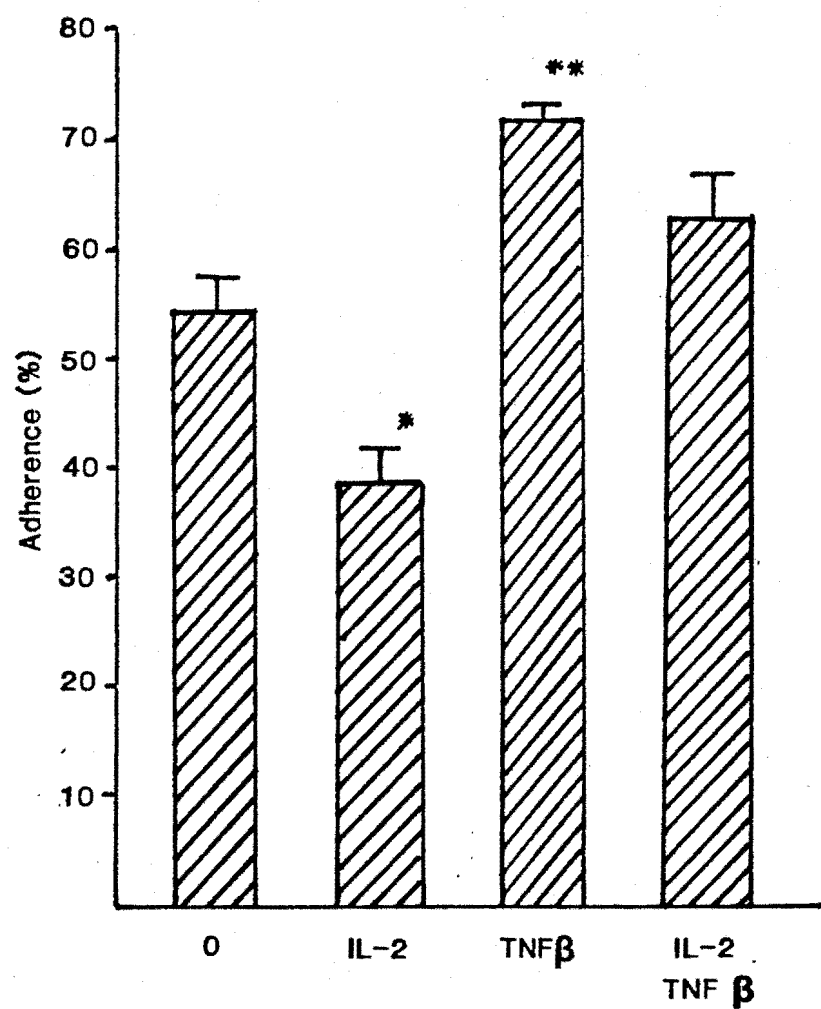


Figure 8.3 Combined effects of IL-2 and TNF β on human neutrophil adherence. Incubation time was 30 min.

* $p < 0.01$

** $p < 0.001$

Table 8.1 Effect of IL-2 and TNF β on FMLP stimulation of neutrophil adherence.

Experimental conditions	Percent adherence (mean \pm S.D.)	P value
1. Control	48.2 \pm 2.8	-
2. FMLP	65.1 \pm 2.3	p < 0.01 (2 vs. 1)
3. IL-2	23.4 \pm 1.5	p < 0.001 (3 vs. 1)
4. IL-2/FMLP	46.0 \pm 2.1	p < 0.001 (4 vs. 2) p < 0.001 (4 vs. 3)
5. TNF β	63.2 \pm 0.8	p < 0.001 (5 vs. 1)
6. TNF β /FMLP	75.7 \pm 2.5	p < 0.01 (6 vs. 5) p < 0.01 (6 vs. 2)

Separate aliquots of neutrophils from a single donor were incubated with either IL-2 (200 U/ml), TNF β (500 U/ml) and combination of FMLP (4×10^{-7} M) with either lymphokine. Incubation time with lymphokine and FMLP was 30 min and 15 min, respectively.

either substance alone (Table 8.1).

Effect of PMA

Phorbol myristate acetate (PMA, Sigma Chemical Company, St. Louis), is a croton oil derivative which activates neutrophils by way of the enzyme protein kinase C (Barrowman et al, 1986) bypassing cell membrane receptors and therefore quite different from the receptor-ligand mechanism of FMLP (Dahlgren et al, 1982; Williams & Cole, 1981).

For these experiments we used the final concentration of 0.1 $\mu\text{g/ml}$ of PMA previously shown to enhance neutrophil adherence (Thong & Currell, 1983). It can be observed from Table 8.2 that IL-2 retards to some extent the activation of adherence by PMA; thus PMA increased adherence to 79.7 ± 1.9 percent from a baseline of 52.6 ± 1.9 percent, but in the presence of IL-2, adherence was increased to only 69.7 ± 1.6 percent.

In contrast, the presence of $\text{TNF-}\beta$ caused adherence to be enhanced to a value of 88.5 ± 0.6 percent, significantly higher than either PMA (79.7 ± 1.9 percent) or $\text{TNF-}\beta$ (66.9 ± 4.1) by themselves.

Heat treatment

We next subjected the IL-2 and $\text{TNF-}\beta$ to heating at

Table 8.2 Effect of IL-2 and TNF β on PMA stimulation of neutrophil adherence.

Experimental conditions	Percent adherence (mean \pm S.D.)	P value
1. Control	52.6 \pm 1.9	-
2. PMA	79.7 \pm 1.9	< 0.001 (2 vs. 1)
3. IL-2	34.1 \pm 5.3	< 0.01 (3 vs. 1)
4. IL-2/PMA	69.7 \pm 1.6	< 0.01 (4 vs. 2) < 0.001 (4 vs. 3)
5. TNF β	66.9 \pm 4.1	< 0.01 (5 vs. 1)
6. TNF β /PMA	88.5 \pm 0.6	< 0.001 (6 vs. 5) < 0.01 (6 vs. 2)

Separate aliquots of neutrophils from a single donor were incubated with either IL-2 (200 U/ml), TNF β (500 U/ml) or combination of PMA with either lymphokine. Incubation time with lymphokine and PMA was 30 min and 15 min, respectively.

80°C for 30 min. It can be seen in Table 8.3 that the effects of these lymphokines on neutrophil adherence are heat-sensitive. The suppressive effect of IL-2 on neutrophil adherence was reversed by heating, as was the enhancing effect of TNF- β .

Polymyxin B treatment

Polymyxin B (PMB) has been shown to inactivate endotoxin, a frequent contaminant of biological specimens (Spear & Teoderescu, 1984). We next pretreated IL-2 and TNF- β with 20 μ g/ml of PMB (Sigma Chemical Company, St. Louis) for 30 min. at 37°C, then incubated each with neutrophils for another 15 minutes.

The results are presented in Table 8.4. PMB suppressed neutrophil adherence as shown in previous studies (Li et al, 1985). Again, IL-2 significantly suppressed neutrophil adherence, while the combination of IL-2 and PMB caused depression of neutrophil adherence greater than each by itself.

TNF- β was again shown to enhance neutrophil adherence. The combination of TNF- β and PMB caused some reduction in neutrophil adherence, but the value of 53.2 ± 4.9 was much higher than that of 18.5 ± 2.5 in neutrophils treated with PMB alone ($p < 0.001$). If the effects of IL-2 or TNF- β on neutrophil adherence were

Table 8.3 Effect of heat treatment of IL-2 and TNF β on human neutrophil adherence.

Experimental conditions	Percent adherence (mean \pm S.D.)
Control (medium only)	49.9 \pm 2.6
IL-2	33.6 \pm 4.6*
IL-2 (80°C/30 min)	47.5 \pm 4.2
TNF β	64.9 \pm 3.3*
TNF β (80°C/30 min)	46.8 \pm 1.9

Separate aliquots of IL-2 and TNF β were heated at 80°C for 30 min. prior to experiments. Final concentrations of IL-2 and TNF β were 200 U/ml and 500 U/ml, respectively.

* $p < 0.01$

Table 8.4 Effect of pretreatment of IL-2 and TNF β with polymyxin B.

Experimental conditions	Percent adherence (mean \pm S.D.)	P value	
		vs. Control	vs. PMB control
Control	42.4 \pm 5.6	—	—
PMB Control	18.5 \pm 2.5	< 0.01	—
IL-2	17.1 \pm 6.7	< 0.01	N.S.
IL-2/PMB	7.5 \pm 7.8	< 0.01	< 0.01
TNF β	60.5 \pm 2.7	< 0.01	< 0.001
TNF β /PMB	53.2 \pm 4.9	< 0.05	< 0.001

Separate aliquots of IL-2 and TNF β were pretreated with PMB for 30 min prior to experiments. Final concentrations of IL-2, TNF β and PMB were 200 units/ml, 500 units/ml and 20 μ g/ml, respectively.

caused by endotoxin contamination, it would be expected that PMB would neutralize their effects but this was not the case.

Discussion

IL-2, also known as T-cell growth factor, is a glycoprotein hormone of 15,000 MW, secreted by T lymphocytes (Smith, 1984; Dinarello & Meir, 1986). Its biological significance became apparent with the discovery of its capacity to expand T cell clones and stimulate cytotoxic lymphocytes for cancer immunotherapy (Rosenberg & Lotze, 1986). Two forms of human TNF have been identified. TNF- α , or cachectin, is a product of macrophages with widespread effects on many tissues (Tracey et al, 1986). TNF- β , which shares 50% homology in nucleotide sequence with TNF- α , is a lymphocyte derivative formerly designated as lymphotoxin (Gray et al, 1984).

Although interferons are known to stimulate certain neutrophil functions (Ferrante & Rencis, 1984; Hokland & Berg, 1981; Shalaby et al, 1985; Seow & Thong, 1986a), little is known about the effects of IL-2 and TNF- β on human neutrophils. TNF- β has been shown to enhance phagocytosis and neutrophil antibody-dependent cell-mediated cytotoxicity (Shalaby et al, 1985). It is

pertinent to note here that the macrophage product, $\text{TNF-}\alpha$ has been reported to cause enhancement of neutrophil adherence (Gamble et al, 1985). The results of the present study showed enhancement of human neutrophil adherence by $\text{TNF-}\beta$ but suppression by IL-2. The opposite effects of these two lymphokines were consistent throughout the experiments. The effects were observed over a range of concentrations similar to their other biological activities, occurred within 15 min. and remained for up to 60 min. Experiments involving simultaneous addition of the lymphokines to neutrophils confirmed the opposing effects of the chemotactic peptide FMLP. The combination of $\text{TNF-}\beta$ and FMLP produced enhancement of neutrophil adherence which exceeded that of either agent alone. Similar results were obtained with the phorbol ester stimulant PMA. These effects were not the result of endotoxin contamination as demonstrated by their sensitivities to heat-inactivation and insensitivities to polymyxin B treatment. Moreover, it is unlikely that the depressive effects of IL-2 can be attributed to endotoxin contamination since endotoxin stimulates rather than inhibits neutrophil functions.

These results indicate that communication between lymphocytes and neutrophils is mediated at least in part by means of lymphokines, including IL-2 and $\text{TNF-}\beta$, and further underlines the central role of T cells in the

regulation of immunological and inflammatory responses. Of the three well-characterised lymphokines that have been studied, two (IFN γ and TNF- β) are up-regulatory and one (IL-2) down-regulatory for neutrophils, and the relative mix and concentrations of these and other cytokines may partially determine the immunological transition from acute to chronic inflammation, or its resolution. Thus, in non-immune individuals or in the early stages of microbial invasion when specific immunological mechanisms are not yet fully mobilised, IFN- γ and TNF- β may promote the recruitment of neutrophils into sites of acute inflammation. Later, the secretion of IL-2 would promote the clonal expansion of lymphocytes and retard the ingress of neutrophils resulting in the lymphocyte predominance which is so characteristic of chronic inflammatory lesions.

Neutrophils are phagocytic cells with important roles in microbial immunity and inflammatory processes. It is therefore not surprising that they are influenced by an array of leukocyte mediators, as shown by this and other studies. There is also increasing evidence to suggest that they are capable of influencing other leukocytes (Yamazaki & Ziff, 1977; Yoshinaga et al, 1980), as well as each other (Seow & Thong, 1986b)

CHAPTER NINE

**MACROPHAGE-NEUTROPHIL INTERACTIONS: CONTRASTING EFFECTS
OF THE MONOKINES, INTERLEUKIN-1 AND TUMOUR NECROSIS
FACTOR (CACHECTIN) ON HUMAN NEUTROPHIL ADHERENCE**

Introduction

Besides their important roles in host defence and inflammation, cells of the monocyte-macrophage series have a major role in immunoregulation by way of antigen presentation and monokine secretion (Unanue, 1984). Cytokines released by macrophages, such as interferons, granulocyte-macrophage colony-stimulatory factor (GM-CSF), and tumour necrosis factor α (TNF- α) are also known to modulate the behaviour of neutrophil leukocytes, the other major phagocytic cell of the body (Ferrante & Rencis, 1984; Vadas et al, 1984; Weisbart et al, 1985; Shalaby et al, 1985; Gamble et al, 1985; Seow & Thong, 1986a).

In this report, we studied the effects of two macrophage-derived cytokines interleukin-1 (IL-1)

(Dinarello & Meir, 1986), and human TNF- α (Beutler & Cerami, 1987) on human neutrophil adherence, one of the earliest observable changes in neutrophil behaviour and an obligatory step in the recruitment of these cells to sites of inflammation and infection (Gallin, 1985). The results demonstrate that recombinant human IL-1 and recombinant human TNF- α have opposite effects on neutrophil adherence.

Materials and Methods

Monokines

Recombinant human IL-1 α was obtained from Dr Peter T. Lomedico and Dr Alvin Stern of Hoffman-La Roche, Nutley, New Jersey, USA, and consists of the 154 amino acid carboxy terminal of the 271 amino acid human IL-1 precursor. It was purified from E.coli and has a specific activity of 3×10^6 units/ml when assayed in the standard mouse thymocyte proliferation assay.

Recombinant TNF- α was kindly provided by Dr G. R. Adolf, Ernst-Boehringer Institut, Vienna, Austria. It was purified from E.coli (>99% purity) and has a specific activity of 6×10^7 units/mg when assayed for cytotoxicity against the actinomycin-D treated mouse connective tissue cell-line L929. Endotoxin contamination was 0.125 EU units/ml by the limulus lysate assay.

Neutrophil isolation

Approximately 20ml of heparinised blood was obtained from healthy donors by venepuncture, layered onto Mono-Poly Resolving Medium (Flow Laboratories, Virginia), (Ferrante & Thong, 1980; 1982) and centrifuged at 400G for 30 min. This one-step technique resulted in the formation of two leukocyte bands at the interface. Neutrophils were removed from the second band, washed twice, resuspended in medium 199, and adjusted to a concentration of $8-10 \times 10^6$ cells/ml. they were of >97% purity.

Neutrophil adherence

This microassay was performed as previously described (Thong & Currell, 1983). Briefly, microcolumns were prepared by carefully weighing out 10mg lots of dacron fibre (Olympic General Products, Qld) and evenly packing them into 100 μ l disposable pipette tips (Stockwell Scientific, Calif), so as to occupy the middle 2cm of the 5cm length. Each microcolumn so prepared can easily accommodate 0.1ml of the neutrophil suspension.

Equal volumes of neutrophil suspension and lymphokine solution were mixed and incubated at 37°C for varying periods of time (incubation time) before

delivery into the microcolumns. The microcolumns were then placed into an incubator at 37°C in humidified air for 5 min. The fluid was extracted into disposable test tubes by a vacuum pump operating at a suction pressure of 250 mbar applied for 1 min. The concentration of neutrophils was determined by Neubauer haemocytometer, and the results calculated as follows:

$$\text{Percent adherence} = 100 - \frac{\text{CE}}{\text{CA}} \times 100$$

where CA is the concentration of neutrophils applied to the microcolumn, and CE the concentration recovered in the effluent.

Experiments were performed in triplicate and results expressed as mean \pm SD. The student's t-test was used for statistical analysis.

Results

Dose response

For these experiments, neutrophils were incubated with various concentrations of IL-1 α or TNF- α at 37°C for 30 min. prior to assay of adherence. The results (Figure 9.1) show that IL-1 α significantly depressed adherence at concentrations of 100 U/ml and 200 U/ml,

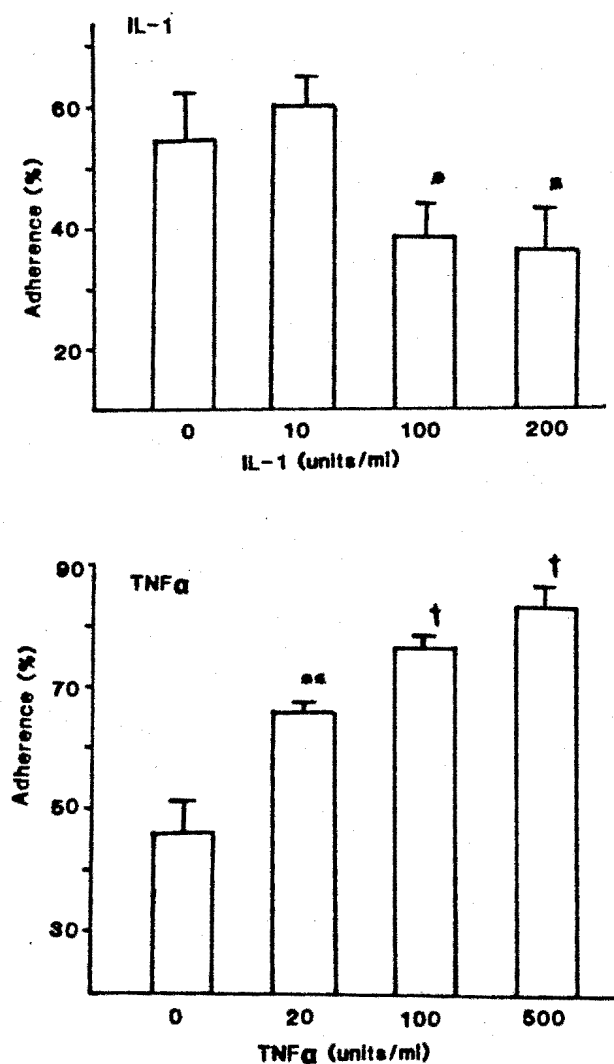


Figure 9.1 Effect of varying concentrations of IL-1 and TNF α on human neutrophil adherence. Various concentrations of monokine were incubated with neutrophils for 30 min at 37°C prior to adherence assay.

* $p < 0.05$

** $p < 0.02$

† $p < 0.01$

but not at 10 U/ml. In contrast, TNF- α significantly enhanced neutrophil adherence at all concentrations used in the experiment (Figure 9.1).

Time course

Neutrophils were pre-incubated with either IL-1 (500 U/ml) or TNF- α (500 U/ml) for various times at 37°C prior to assay of adherence. The results show that for both IL-1 α and TNF- α , modulation of neutrophil adherence was evident as early as 5 min. after interaction with the monokines, and that their effects were sustained for at least 60 min. (Figure 9.2) ($p < 0.01$).

Combined effects

IL-1 α (500 U/ml) and TNF- α (500 U/ml) were added either alone or in combination to neutrophils 30 min. prior to measurement of adherence. The results (Figure 9.3) showed that simultaneous addition of IL-1 α and TNF- α resulted in intermediate values of adherence, between the significant depression seen with IL-1 α and the significant augmentation seen with TNF- α .

Effect on FMLP stimulation

The chemotactic peptide Formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma Chemical Co, St. Louis) stimulated neutrophil behaviour by binding to specific receptors on the cell surface (Weisbart et al, 1986).

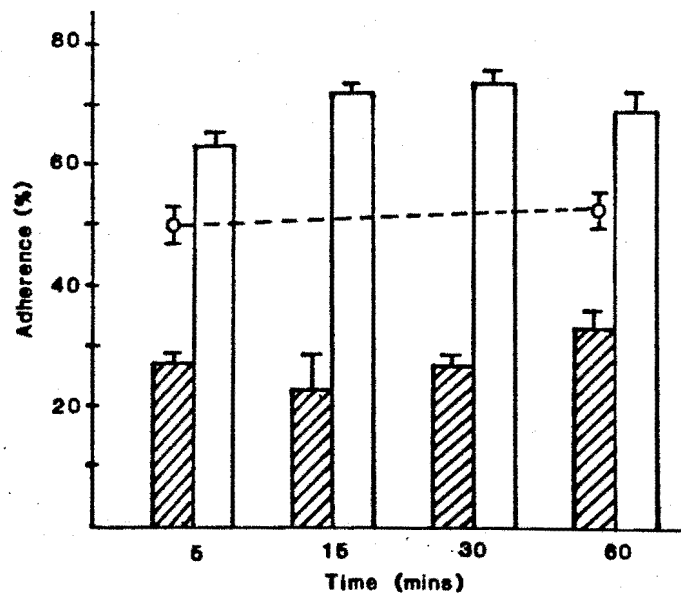


Figure 9.2 Time course of the effects of IL-1 (▨) and TNF α (□) on human neutrophil adherence (○ Control). Neutrophils were pre-incubated with either monokine for varying time periods prior to assay of adherence.

* $p < 0.01$ for all values.

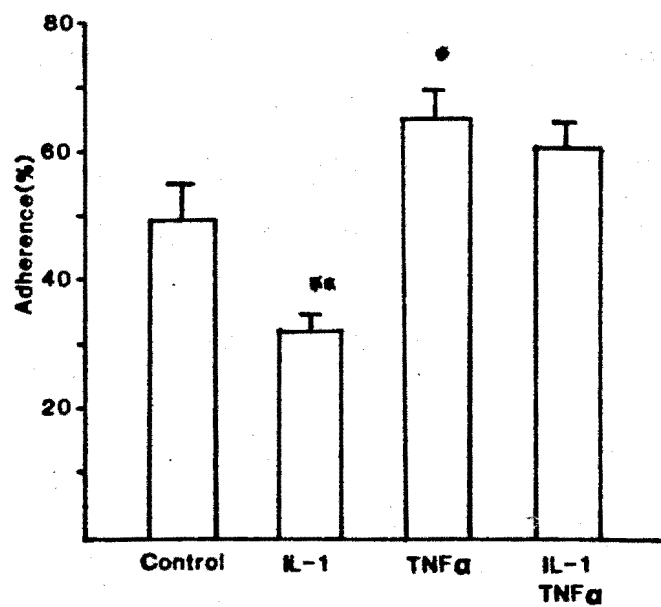


Figure 9.3 Simultaneous addition of IL-1 and TNFα on neutrophil adherence. The monokines were added to neutrophils singly or in combination 30 min prior to assay of adherence.

* $p < 0.05$

** $p < 0.01$

Pre-treatment of neutrophils by IL-1 α (500 U/ml) for 30 min. prevented the full augmentation of neutrophil adherence by FMLP (Figure 9.4). By contrast, pre-treatment of TNF- α (500 U/ml) for 30 min. augmented the adherence of FMLP-stimulated neutrophil adherence.

Effect on PMA stimulation

The tumour promotor phorbol myristate acetate (PMA, Sigma Chemical Co, St. Louis), is a potent stimulant of neutrophils by direct activation of protein kinase C (Nishizuka, 1984). In this experiment, we pre-treated neutrophils with IL-1 α (500 U/ml) and TNF- α (500 U/ml) to see whether these monokines would modify the stimulant effects of PMA. The results (Table 9.1) show that IL-1 α reduced the enhancement of neutrophil adherence caused by PMA ($p < 0.001$); while TNF- α augmented PMA enhanced adherence ($p < 0.01$).

Effect of heating

Heating at 80°C for 30 min. completely abolished the modulatory effects of IL-1 α and TNF- α on neutrophil adherence (Figure 9.5).

Effect of polymyxin B

The biological properties of bacterial lipopolysaccharide (LPS) can be abolished by treatment with

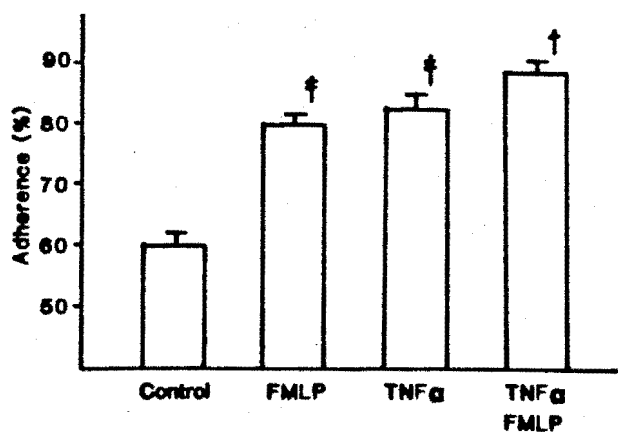
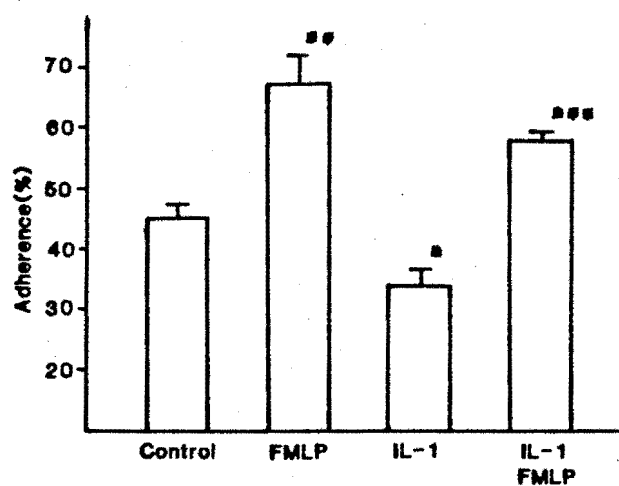


Figure 9.4 Effect of IL-1 and TNF α on the FMLP-mediated stimulation of neutrophil adherence. Neutrophils were pre-treated with monokine for 15 min prior to the addition of FMLP for another 15 min.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.02$ vs. control,
 < 0.05 vs. FMLP

‡ $p < 0.001$

† $p < 0.001$ vs. control,
 < 0.05 vs. FMLP

Table 9.1 Effect of IL-1 and TNF α on PMA-stimulation of human neutrophil adherence.

Experimental conditions	Percent adherence (mean \pm S.D.)	P value
1. Control	49.1 \pm 5.8	—
2. PMA	86.7 \pm 1.5	< 0.001 (2 vs. 1)
3. IL-1	28.8 \pm 5.9	< 0.05 (3 vs. 1)
4. IL-1 & PMA	71.5 \pm 2.3	< 0.001 (4 vs. 2) < 0.001 (4 vs. 3)
5. TNF α	81.2 \pm 2.3	< 0.001 (5 vs. 1)
6. TNF α & PMA	92.4 \pm 1.1	< 0.02 (6 vs. 2) < 0.01 (6 vs. 5)

Separate aliquots of neutrophils from a single donor were incubated with either IL-1 (200 U/ml), TNF α (500 U/ml), PMA (0.1 μ g/ml) or combination of PMA with either monokine. Incubation time with monokine and PMA was 30 min and 15 min, respectively.

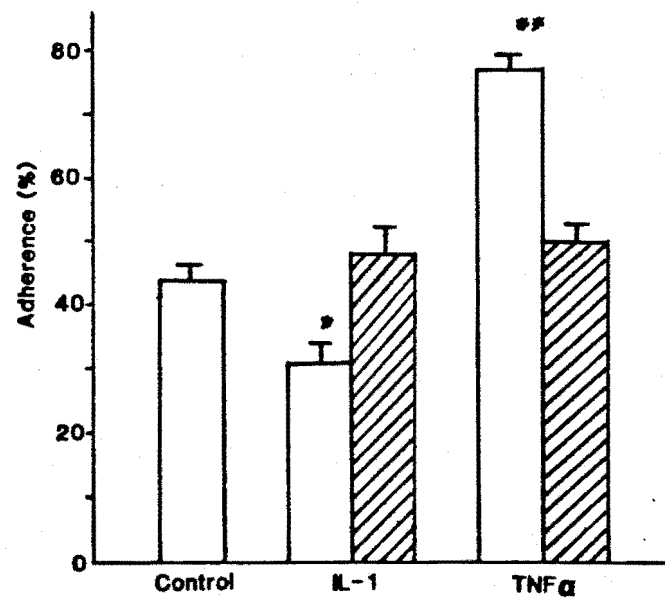


Figure 9.5 Effect of heat treatment of IL-1 and TNF α on human neutrophil adherence. The monokines were heated at 80°C for 30 min and added to neutrophils for another 15 min prior to assay of adherence (□ unheated, ▨ heated).

* $p < 0.05$

** $p < 0.001$

polymyxin B (PMB, Sigma Chemical Co, St. Louis) (Spear & Teodorescu, 1984). We used PMB to examine whether LPS (as a possible contaminant) was responsible for the cytokine activity on neutrophils. The results show that pre-treatment of IL-1 α with 5 μ g/ml of PMB did not prevent suppression of neutrophil adherence by IL-1 (Table 9.2). Similar treatment of TNF- α had no effect on the ability of this cytokine to enhance neutrophil adherence (Table 9.2). PMB at the concentration used in these experiments has no effect per se on neutrophil adherence (Li et al, 1985).

Discussion

The cytokine IL-1, a protein of molecular weight 17,000 daltons, appears to have a multiplicity of functions which is revealed by acronyms used over the last decade: lymphocyte activating factor, mitogenic protein, B-cell activating factor, B-cell differentiating factor, T-cell replacing factor, endogenous or leukocyte pyrogen, leukocyte endogenous mediator, and monocyte cell factor (Billiau et al, 1985; Dinarello & Meir, 1986). Besides its effects on lymphoid cells, it is clear that IL-1 has significant effects on non-lymphoid cells as well (Dinarello & Meir, 1986). It is therefore not surprising that we found IL-1 to affect neutrophil adherence, although the finding that it

Table 9.2 Effect of pretreatment of IL-1 and TNF α with polymyxin B.

Experimental conditions	Percent adherence (mean \pm S.D.)	P value
1. Control	44.9 \pm 2.4	—
2. PMB	42.7 \pm 2.7	> 0.1 (2 vs. 1)
3. IL-1	28.6 \pm 3.9	< 0.01 (3 vs. 1)
4. IL-1 & PMB	28.6 \pm 3.1	< 0.01 (4 vs. 2) > 0.1 (4 vs. 3)
5. TNF α	69.1 \pm 2.1	< 0.001 (5 vs. 1)
6. TNF α & PMB	67.2 \pm 2.8	< 0.001 (6 vs. 2) > 0.1 (6 vs. 5)

Separate aliquots of monokines were preincubated with polymyxin B (5 μ g/ml) for 30 min and then incubated with neutrophils from a single donor for 15 min prior to assay of adherence.

actually suppresses neutrophil adherence is both interesting and unexpected. The molecule exists in at least two forms of pI5 (IL-1 α) and pI7 (IL-1 β). In the present study, we examined the effects of IL-1 by using a recombinant human IL-1. We found activity at 100 U/ml or greater, but not at 10 U/ml, perhaps because recombinant molecules tend to be less potent than glycosylated natural molecules. The results demonstrate that the suppressive effects of IL-1 on neutrophil adherence were not due to LPS (as possible contaminant), as shown by abolition of IL-1 activity by heating since LPS is resistant to heat (Hibbs et al, 1977) and preservation of IL-1 activity after PMB treatment, which has been shown to inactivate LPS (Spear & Teodorescu, 1984).

Our results also demonstrate that IL-1 has the capacity to suppress the augmented neutrophil adherence induced by exogenous stimuli such as FMLP, which binds to surface membrane receptors, and suggest that IL-1 and possibly other IL-1 species can influence the deployment of neutrophils during the course of inflammation or infection. Of further interest was the finding of enhancement by TNF- α , as both types of cytokine can be released by macrophages during inflammation at the same site. In this regard, it was found that the addition of both IL-1 and TNF- α resulted in neutrophil adherence of intermediate degree, between the depression caused by

IL-1 and the enhancement caused by TNF- α

The effects of stimuli such as PMA, which bypasses cell surface receptors and activates neutrophils directly via protein kinase C, was also found to be influenced by these cytokines. Thus, IL-1 was found to ameliorate the stimulatory effect of this phorbol ester, while TNF- α augmented the stimulatory effect.

Our data confirm previously reported findings that TNF enhances a range of neutrophil activities, including adherence (Shalaby et al, 1985; Gamble et al, 1985). The effects of IL-1 on neutrophils is less clear. Smith et al (1986) found that IL-1 stimulated neutrophil degranulation, while Pincus et al (1986) recently reported that IL-1 inhibited the oxidative metabolism of human eosinophils. Thus, IL-1 should not be regarded as purely an activator of cellular function, and our data would support this contention. Furthermore, IL-1 does not have the cachectic activity of TNF- α and TNF- α is lacking in Lymphocyte Activating Factor activity. Apart from these differences, IL-1 and TNF- α are concordant in most of their other biological properties, including thermoregulation, cytotoxic activity for tumour cells, Osteoclast Activating Factor activity, and stimulation of synovial cell production of prostaglandin E₂, and collagenase (Onozaki et al, 1985; Dinarello & Meir,

1986; Mannell, 1986; Beutler & Cerami, 1987).

The precise molecular components which dictate events in the evolution of the inflammatory process, from the initial preponderance of neutrophils to the later influx of mononuclear cells, is poorly understood but our data suggest that the relative mix of up-regulatory (eg. TNF- α) and down-regulatory (eg. IL-1) monokines present in the inflammatory lesion may partly determine the continuing recruitment of neutrophils or their dissipation.

CHAPTER TEN

INHIBITORY EFFECTS OF TETRANDRINE ON HUMAN NEUTROPHIL
AND
MONOCYTE ADHERENCE

Introduction

Progressive pulmonary fibrosis produced by the inhalation of silica, asbestos, coal and other mineral dusts constitutes an important health hazard in many occupations (Berlinger, 1982). Treatment for these pneumoconioses is at present unsatisfactory, but recent reports from China indicate that the herbal alkaloid, tetrandrine, can not only retard, but also halt and sometimes reverse the pulmonary lesions of silicosis (Li et al, 1981; Yu et al, 1983; Liu et al, 1983). Since conventional anti-inflammatory, cytotoxic and immunosuppressive drugs are ineffective in silicosis, it is reasonable to assume that tetrandrine may have unique and interesting properties.

Tetrandrine was originally isolated from the ancient Chinese remedy for rheumatoid arthritis known as Han-fang-chi, the tuberous root of a creeper with the botanical name of *Stephania tetrandra* S. Moore (Li et al, 1981; Yu et al, 1983; Liu et al, 1983). It is an alkaloid of molecular weight of 622.73. Its empirical formula is $C_{38}H_{42}O_6N_2$, and its structural formula is shown in Fig. 10.1.

The mode of action of tetrandrine in silicosis is poorly understood. Since phagocytic cells have a central role in the pathogenesis of silicosis (Allison & Morgan, 1979; DeShazo, 1982; Doll et al, 1983), we postulate that tetrandrine may achieve its beneficial effects in this disease by suppression of phagocytic cells. In the present study, we examined the effects of tetrandrine on human monocyte and neutrophil adherence. The adherence of phagocytic cells to vascular endothelium is a prerequisite for their diapedesis into the perivascular compartment and their subsequent accumulation at sites of inflammation (Cohnheim, 1882; Fehr & Dahiden, 1979; Crowley et al, 1980; Dana et al, 1984).

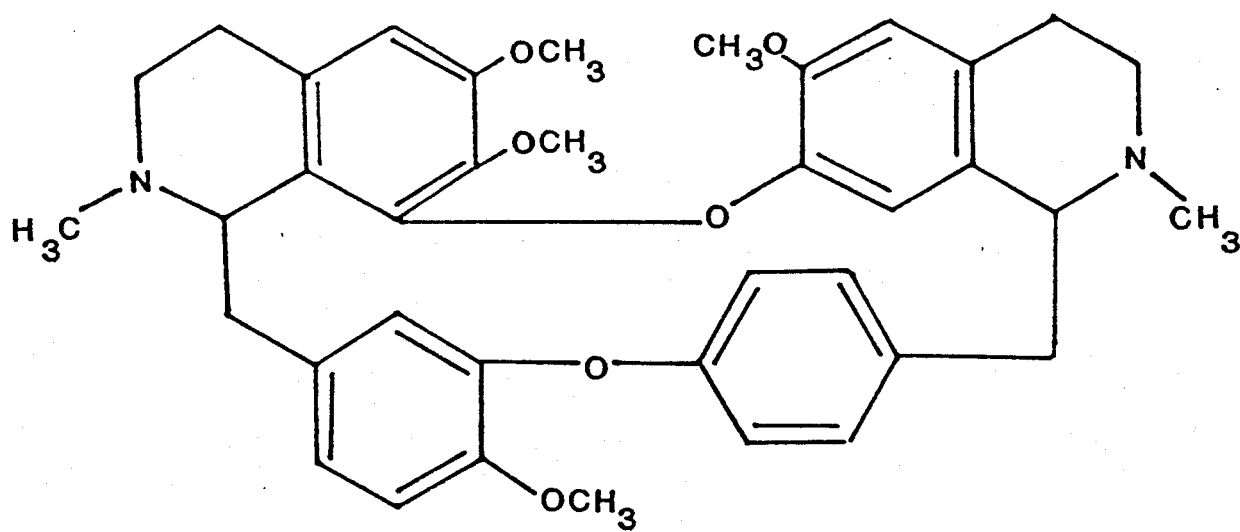


Figure 10.1 The Chemical Structure of Tetrandrine.

Materials and Methods

Tetrandrine

A purified preparation of tetrandrine (>98% purity) was obtained from the Yichang Pharmaceutical Factory, Beijing, People's Republic of China. It was dissolved in pure ethanol, and further dilutions made from the stock solution in either medium 199, or in glucose-free Dulbecco's balanced salt solution (BSS). Preliminary experiments indicate that the final concentration of 2.5% alcohol did not affect phagocyte function; this is consistent with other reports of the effects of ethanol on phagocyte functions (MacGregor et al, 1974).

Isolation of neutrophils and monocytes

About 30 ml of heparinised blood was obtained from each healthy volunteer by venepuncture. Centrifugation on Mono-Poly Resolving Medium (Flow Laboratories, Virginia, USA) at 1000G for 30 min. resulted in separation of neutrophils (>97% purity) on the second band (Ferrante & Thong, 1980). Monocytes from the top band were further enriched to >93% purity by centrifugation on percoll gradients (Giddings et al, 1980). The cells were washed twice and resuspended in either medium 199 or Dulbecco's BSS depending on experiment.

Neutrophil and monocyte adherence

The nylon fibre microcolumn assay for the neutrophil and monocyte adherence was performed as previously described (Thong & Currell, 1983). Briefly, the nylon fibre microcolumns were prepared by carefully weighing out 10 mg lots of teased nylon fibre (Olympic Products, Queensland, Australia). These were placed into 100 μ l disposable pipette tips (Stockwell Scientific, Monterey Park, Calif) so as to occupy the centre 2cm portion of the 5cm pipette tip. Neutrophil or monocyte suspensions, with or without tetrandrine, were adjusted to concentrations between $4-6 \times 10^6$ cells/ml, and 100 μ l was delivered into each nylon fibre microcolumn. After incubation for 10 min. (neutrophils) or 15 min. (monocytes) at 37°C and high humidity in order to allow for contact between cells and nylon fibre, the microcolumns were placed in a specially designed apparatus (Thong & Currell, 1983), and the fluid extracted by a vacuum suction pressure of 250 mbar applied for 1 min. into disposable test tubes. The concentrations of cells were determined by Neubauer Counting Chamber, and the results calculated as follows:

$$\text{Percent adherence} = 100 - \frac{\text{Neutrophil conc. in effluent} \times 100}{\text{Neutrophil conc. in original suspension}}$$

In some experiments. the tumour promotor, phorbol myristate acetate (PMA, Sigma Chemical Company, St. Louis USA) was used to augment neutrophil and monocyte adherence.

Deoxyglucose uptake

We used a newly developed deoxyglucose uptake microassay for these experiments (Seow et al, 1987e). Approximately 5×10^5 cells in 0.1 ml of Dulbecco's BSS was delivered to each well of a round bottom microtitre plate. Another 0.05 ml of Dulbecco's BSS containing tetrandrine was added to test wells. Control wells received Dulbecco's BSS without tetrandrine. A further 0.05ml of Dulbecco's BSS containing 2-D³H-deoxyglucose (Radiochemical Centre, Amersham, UK) was added to each well to give a final concentration of 0.78 μ Ci/ml. The microtitre plate was incubated at 37°C in a humidified air atmosphere for 30 mins, centrifuged at 4°C and 800G for 5 mins, and 0.05ml of supernatant removed for determination of radioactivity in a LKB liquid scintillation counter. The uptake of ³H-deoxyglucose is calculated from this formula:

$$\text{Deoxyglucose uptake} = \text{Total DPM added} - \text{DPM supernatant}$$

Experiments were performed in triplicate and results expressed as mean DPM \pm SD.

Cell viability

This was assessed by trypan blue dye exclusion as follows (Ferrante et al, 1979): 0.1 ml of cell suspension was mixed with 0.1 ml of 0.2% trypan blue solution, incubated at 37°C for 5 mins, and the number of stained cells determined by counting in a haemocytometer.

Statistical analysis

The student's t-test was used for statistical analysis.

Results

Effect of tetrandrine on adherence

We first determined whether tetrandrine had any effect on neutrophil and monocyte adherence. The results showed suppression of both neutrophils and monocytes by tetrandrine, with monocytes being more sensitive to the effects of this drug (Table 10.1). Although tetrandrine suppressed neutrophil adherence in dose-dependent fashion, suppression was only significant at the concentration of 10 µg/ml, reducing adherence from $66.7 \pm 4.3\%$ to $37.6 \pm 5.9\%$ in one experiment ($p < 0.01$), and from $58.7 \pm 5.6\%$ to $30.9 \pm 4.7\%$ in another ($p < 0.01$). For monocytes however, significant inhibition of adherence was evident with concentrations of tetrandrine as low as 0.1 µg/ml (Table 10.1)

Table 10.1 Inhibition of Neutrophil and Monocyte Adherence by tetrandrine: dose-response relationships.

Tetrandrine conc. ($\mu\text{g/ml}$)	% Adherence (mean \pm S.D.)			
	Exp. 1		Exp. 2	
	Neutrophils	Monocytes	Neutrophils	Monocytes
0	66.7 \pm 4.3	59.4 \pm 2.3	58.7 \pm 5.6	85.0 \pm 0.5
0.1	64.8 \pm 4.9	43.5 \pm 3.0*	58.6 \pm 6.1	71.1 \pm 2.8*
1.0	58.4 \pm 9.2	43.0 \pm 5.1*	51.4 \pm 3.7	53.6 \pm 9.5**
10.0	37.6 \pm 5.9*	20.9 \pm 10.5*	30.9 \pm 4.7*	26.4 \pm 5.4**

Each experiment was performed with cells from different donors. Cells were incubated for 30 mins with varying concentrations of tetrandrine prior to adherence microassay. Results are expressed as mean \pm S.D. of triplicate samples.

* $p < 0.01$

** $p < 0.001$

In the next set of experiments, we sought to determine whether tetrandrine binds irreversibly to cell membranes or subcellular structures. Neutrophils or monocytes from a single donor were divided into three lots. One lot was incubated with 10 μ g/ml of tetrandrine for 30 mins, washed thrice, and resuspended in medium 199 for assay of adherence. Another lot was similarly treated with tetrandrine, but not washed. The third lot was not exposed to tetrandrine and served as control. The results showed that washing completely abrogated the inhibitory effect of tetrandrine on both neutrophils and monocytes (Table 10.2). These findings were confirmed in a second set of experiments using a different donor.

The tumour promoter, PMA, is a potent stimulant of phagocyte function, including adherence (O'Flaherty et al, 1979). We wondered whether tetrandrine would be able to reduce or reverse the augmentation of adherence induced by PMA. The results showed that PMA caused significant enhancement of adherence in the absence of tetrandrine. By contrast, tetrandrine at 10 μ g/ml completely abolished this effect (Figure 10.2). For neutrophils, tetrandrine reduced adherence from $78.2 \pm 4.1\%$ to $64.5 \pm 6.3\%$ ($p < 0.05$) in samples without PMA; in PMA-stimulated samples, adherence was reduced from $84.6 \pm 1.3\%$ to $65.4 \pm 1.3\%$ ($p < 0.001$).

Table 10.2 Reversibility of Inhibition of Neutrophil and Monocyte Adherence by Tetrandrine.

Manipulation	% Adherence (mean \pm S.D.)			
	Exp. 1		Exp. 2	
	Neutrophils	Monocytes	Neutrophils	Monocytes
Control	84.9 \pm 2.4	81.9 \pm 3.0	65.2 \pm 3.9	43.3 \pm 6.8
Unwashed	41.6 \pm 4.7**	73.4 \pm 1.2†	41.8 \pm 5.6*	26.3 \pm 2.7†
Washed	83.3 \pm 3.3	82.9 \pm 1.2	64.3 \pm 6.7	48.5 \pm 2.9

Neutrophils or monocytes were incubated with 10 μ g/ml of tetrandrine for 30 min, washed twice and resuspended in medium 199. Results are expressed as mean \pm S.D. of triplicate samples. Each experiment was performed with cells from different donors.

* $p < 0.01$

** $p < 0.001$

† $p < 0.02$

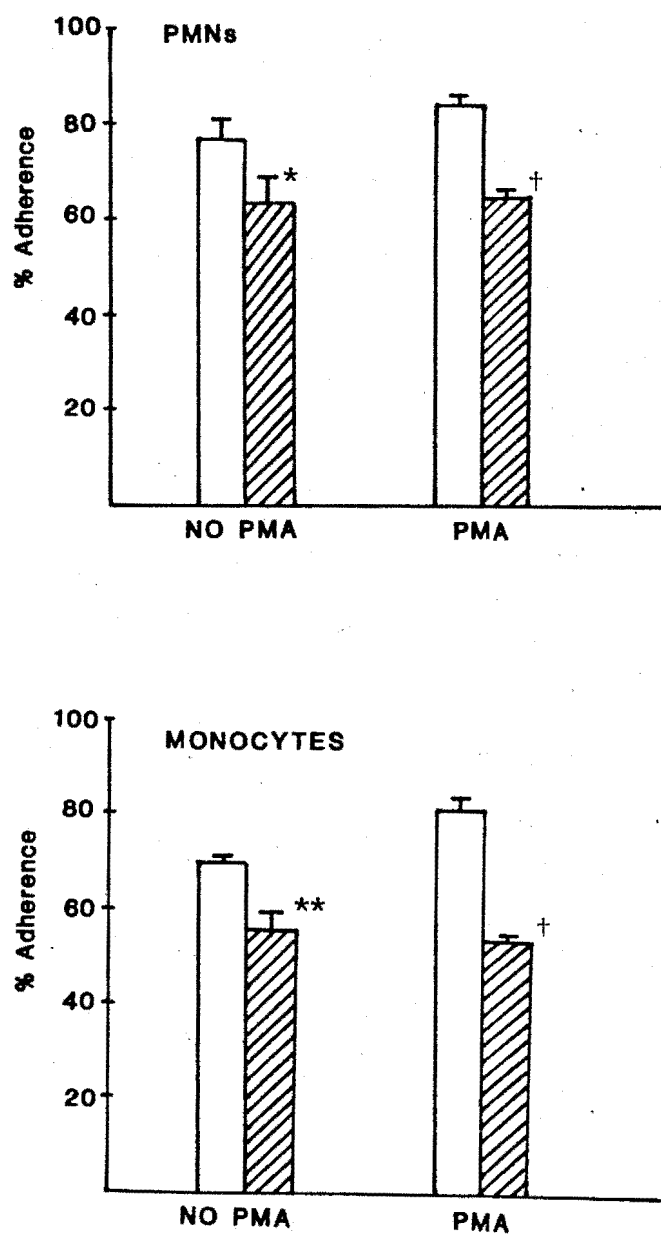


Figure 10.2 Suppression of PMA-induced augmentation of neutrophil and monocyte adherence by tetrandrine. Results are expressed as mean \pm S.D. of triplicate samples. \square No tetrandrine, ▨ 10 μg tetrandrine. Significant suppression of adherence by tetrandrine, * $p < 0.05$, ** $p < 0.01$, + $p < 0.001$.

Similarly, monocyte adherence in samples without PMA was reduced from $69.2 \pm 1.0\%$ to $55.3 \pm 3.9\%$ ($p < 0.01$). PMA increased monocyte adherence in this donor to $81.1 \pm 2.3\%$, but this was reduced to only $53.2 \pm 1.2\%$ ($p < 0.001$) by tetrandrine at 10 ug/ml.

Effect of tetrandrine on deoxyglucose uptake

Energy for adherence in phagocytic cells is provided by glucose metabolism via the glycolytic pathway (Kelly & Thong, 1984; Grinell & Srere, 1971). One way in which tetrandrine can inhibit neutrophil and monocyte adherence may be by interfering with energy requirements. This possibility can be investigated by measuring the cellular accumulation of ^3H -deoxyglucose, an analogue of glucose which can be taken up but not metabolised (McCormack et al, 1981). The results (Table 10.3) show that tetrandrine produced significant suppression of ^3H -deoxyglucose uptake by both neutrophils and monocytes ($p < 0.001$).

Cell viability studies

Neutrophils and monocytes were separately incubated with 10 ug/ml of tetrandrine for 30 min, before the addition of trypan blue (Ferrante et al, 1979). The results showed $>97\%$ viability, similar to controls without tetrandrine.

Table 10.3 Tetrandrine-induced suppression of ^3H -deoxyglucose uptake by neutrophils and monocytes.

Tetrandrine concentration $\mu\text{g/ml}$	^3H -deoxyglucose uptake DPM (mean \pm S.D.)			
	Exp. 1		Exp. 2	
	Neutrophils	Monocytes	Neutrophils	Monocytes
0	41,761 \pm 1,648	34,608 \pm 1,205	28,171 \pm 768	24,720 \pm 405
10	32,482 \pm 833*	22,359 \pm 411*	24,000 \pm 369*	18,673 \pm 533*

Cells were incubated with tetrandrine and ^3H -deoxyglucose for 30 min, centrifuged, and the supernatant removed for determination of glucose-uptake. Results were expressed as mean DPM \pm S.D. of triplicate samples.

* $p < 0.001$

Discussion

Much more is known about the pathogenesis of pneumoconiotic diseases than other chronic inflammatory conditions such as rheumatoid arthritis or connective tissue disease where the initiating events remain enigmatic. In the pneumoconioses, particles of mineral dusts lodged in pulmonary alveoli are ingested by cells of the monocyte-macrophage lineage. As these dust particles are toxic and non-degradable, the macrophages die and release lysosomal enzymes and oxygen free radicals which damage surrounding tissues (Allison & Morgan, 1979; DeShazo, 1982; Doll et al, 1983). They also release monokines which attract neutrophils that contribute to tissue damage by similar mechanisms (Miller & Kagan, 1977; Stankers & Salvaggio, 1981). Other monokines attract monocytes and lymphocytes to the area, so that a vicious cycle is set up. Fibrosis is the end result of tissue injury produced by this chronic inflammatory process.

There was no known treatment for silicosis until reports from China indicated that tetrandrine may have beneficial therapeutic effects on silicosis. One study of 33 patients treated for 3 years demonstrated clinical and radiological improvement in two-thirds, while the

remainder showed retardation of disease activity (Li et al, 1981). Using a rat model of silicosis, other investigators have shown retardation and reversal of pulmonary fibrosis in controlled experiments (Yu et al, 1983; Liu et al, 1983).

The mode of action of tetrandrine in silicosis is not well understood at present. The results of the present study suggest that one of the ways it may produce its therapeutic benefits in silicosis is by interference with recruitment of phagocytic cells. We find that tetrandrine in concentrations (0.1-10 ug/ml) readily achievable in plasma during therapy (Li et al, 1981; Yu et al, 1983; Liu et al, 1983) caused a dose-dependent inhibition of adherence of neutrophils and monocytes. Dye-exclusion studies showed that tetrandrine was non-toxic to these cells at these concentrations. The suppression of adherence was reversible by washing, suggesting that the drug does not bind tightly to membrane components. The ability of the tumour promotor, PMA, to enhance adherence was abolished by tetrandrine. Finally, tetrandrine inhibited the uptake of deoxyglucose, indicating that one mechanism by which it suppresses adherence may be by disrupting cellular energy supplies. As adherence to vascular endothelium constitutes the first and crucial step for phagocytic cells to leave the circulation and congregate at inflammatory foci (Cohnheim, 1882; Fehr & Dahinden,

1979; Crowley et al, 1980; Dana et al, 1984), these results indicate that at least one of the modes of action of tetrandrine in silicosis is by interfering with the recruitment of phagocytic cells. Others have suggested that tetrandrine may work by suppressing fibrogenesis (Liu et al, 1983), but macrophage-derived factors have been shown to induce fibrogenesis in silicosis (Allison & Morgan, 1979; DeShazo, 1982; Doll et al, 1983), so that our findings of marked inhibition of monocyte adherence by tetrandrine tie in well with current thinking about the critical role of these cells in the pathogenesis of silicotic inflammation and fibrosis.

Tetrandrine is worthy of study for a number of reasons. First, a better understanding of the mode of action of tetrandrine can provide a rational basis for clinical treatment. Second, because it appears superior to conventional anti-inflammatory cytotoxic and immunosuppressive agents in silicosis, it may possess unique properties that can form the basis for the development of a new class of anti-inflammatory drugs for the treatment of not only silicosis, but also other inflammatory diseases. Finally, because its chemical structure is known, it and its analogues may serve as useful probes for the study of molecular and cell biology of phagocytes.

CHAPTER ELEVEN

ANTI-PHAGOCYTIC AND ANTI-OXIDANT PROPERTIES OF THE PLANT
ALKALOID TETRANDRINE

Introduction

Silicosis is an occupational lung disease resulting from inhalation of silica dust which causes chronic inflammation and progressive pulmonary fibrosis. Current knowledge of its pathogenesis indicates that silica particles lodged in the alveoli are first ingested by cells of the monocyte-macrophage series. Since silica is toxic and non-degradable, the macrophages release lysosomal enzymes and oxygen-derived free-radicals which damage lung tissue, and cytokines which recruit neutrophils, lymphocytes and more macrophages into the lesion. A vicious cycle is set up, and pulmonary fibrosis is the end result of this chronic inflammatory process (Bateman et al, 1982; Bowden & Adamson, 1984; Doll et al, 1982; Heppleston, 1967).

There was no effective treatment for any of the pneumoconioses (Ziskind et al, 1976) until reports from

China showed that tetrandrine, a plant alkaloid isolated from "hanfangji", a traditional Chinese remedy for rheumatic diseases, can retard, halt and sometimes reverse the fibrotic lesions of silicosis. One study of 33 patients treated for 3 years in an open trial showed clinical and radiological improvement in two-thirds, and retardation of disease activity in the remainder. Toxic or undesirable side-effects were not observed (Li et al, 1981). In controlled studies using a rat model of silicosis, others have shown retardation and reversal of pulmonary fibrosis (Liu et al, 1983; Yu et al, 1983).

Since conventional anti-inflammatory and immunosuppressive drugs are ineffective in the silicosis (Ziskind et al, 1976), it is reasonable to assume that tetrandrine may have unique properties which can form the basis for the development of a new class of anti-inflammatory agents. Its mode of action is poorly understood, but studies have shown that collagen synthesis (Liu et al, 1983) and phagocyte adherence are suppressed by tetrandrine (Seow et al, 1986).

In this report, we describe the effects of tetrandrine on neutrophil leukocytes, important effector cells in silicosis (Weissman et al, 1980) as well as other inflammatory disease.

Materials and Methods

Tetrandrine

Tetrandrine is the major alkaloid in "hanfangji", the root of the creeper *Stephania tetrandra* S. Moore of the *Menispermaceae* family. It is a bisbenzylisoquinoline with a molecular weight of 622.73 and empirical formula $C_{38}H_{42}O_6N_2$. Tetrandrine powder, of >98% purity, was obtained from Yichang Pharmaceutical Company, Hupeh Province, Peoples Republic of China, and a stock solution made up by dissolving in ethanol (2 mg/ml). It was further diluted in medium 199 for the experiments; this low concentration of ethanol (0.5%) has been shown to have no effect on neutrophil functions (MacGregor et al, 1974; Seow et al, 1986a).

Neutrophil isolation

Heparinised blood contained from healthy donors was layered into Mono-Poly Resolving Medium (Flow Laboratories, Sydney), and centrifuged at 600G for 30 mins. The leukocytes resolved into two bands at the interface. The second band was removed, washed twice and resuspended in medium 199 for the experiments. Neutrophils isolated by this one-step procedure were of >97% purity (Ferrante & Thong, 1980).

Neutrophil locomotion

The movement of neutrophils under agarose was performed as previously described (Ferrante et al, 1980; 1986). Briefly, 3ml of 2 x N medium 199 containing 10% heat-inactivated foetal calf serum were mixed with 3ml of 2% agarose solution and poured into 60 x 15mm tissue culture plates. Wells of 2mm diameter and 3mm apart were punched in sets of 3. The middle well received 5 μ l neutrophil suspension at a concentration of 4×10^7 /ml, the outer well received a chemoattractant, and the inner well received medium 199 only. The chemoattractant was made by incubating 1×10^7 *Candida albicans* with 2ml fresh human serum for 30 min. at 37°C, centrifuged at 1,000 G for 10 mins, and the activated serum removed.

The agarose plates were kept in a 5% CO₂-air atmosphere and high humidity for 2 hrs, and the distance moved towards the inner well (random movement) and the outer well (chemotaxis) measured with the aid of an eyepiece grid in an inverted microscope. Results are expressed as mean mm/2hr \pm SD. of triplicate experiments. Neutrophils were incubated with various concentrations of tetrandrine for 15 mins. prior to the experiments. Also, tetrandrine at similar concentrations was incorporated in the agarose plates.

Phagocytosis

A radiometric assay for phagocytosis was performed as previously described (Ferrante & Thong, 1979). Briefly, 2×10^7 *Candida albicans* were mixed with 5×10^6 neutrophils in 1ml of medium 199 containing 2.5% fresh human serum. The 10ml disposable test tubes were placed in a rotatory shaker at 37°C for 30 min, after which 0.1ml of suspension was removed and placed in round-bottom wells of microtitre plates. To each well was added 10 μ l of ^{14}C uridine at a concentration of 0.93 $\mu\text{Ci/ml}$. and the plate incubated at 37°C for 1 hour, centrifuged at 1,000 G for 10 mins, and 0.05 ml of supernatant removed for quantitation of ^{14}C -uridine in a scintillation spectrophotometer. Percentage of *C. albicans* phagocytosed was calculated as follows:

$$\% \text{ phagocytosis} = \frac{T - u}{T} \times 100$$

where T is the total uptake of ^{14}C -uridine by *C. albicans*, and u the ^{14}C -uridine uptake by unphagocytosed *C. albicans*.

Experiments were performed in triplicate and expressed as mean \pm SD. Neutrophils were pre-incubated with tetrandrine at various concentrations for 15 mins prior to experiments.

Degranulation

Two marker enzymes were used to measure neutrophil degranulation: lysozyme, which is present in both azurophil and specific granules, and beta-glucuronidase stored in azurophil granules only.

Briefly, 1×10^7 neutrophils were mixed with either 0.1 $\mu\text{g/ml}$ of phorbol myristate acetate (PMA, Sigma Chemical, St. Louis) or 1×10^{-7} M formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma Chemical, St. Louis) in a final volume of 1ml for 60 mins at 37C. When FMLP was used as the stimulant, the neutrophils were primed with cytochalasin B (5 μl of 0.25 $\mu\text{g/ml}$). The cells were centrifuged at 1,000 G for 10 mins, and the supernatant removed for assay of lysozyme and beta-glucuronidase.

Lysozyme content was measured by the rate of lysis of the yeast *Micrococcus luteus* (Ferrante et al, 1987; West et al, 1974). To each 0.8ml of 2mg/ml of *M. luteus* in a spectrophotometer cuvette was added 0.1ml of supernatant. The contents were mixed and the rate of change in absorbance at 450nm followed immediately.

Beta-glucuronidase content was determined either by spectrophotometry using phenolphthalein glucuronic acid as substrate (Gallin et al, 1982) or by fluorimetry using

4-methylumbelliferyl -D-glucuronide as substrate (Kolodney, 1976).

Experiments were performed in triplicate and results expressed as percentage release of lysosomal enzyme (mean \pm SD). Neutrophils were incubated with varying concentrations of tetrandrine for 15 min. prior to experiments.

Respiratory burst

Stimulated neutrophils metabolise glucose via the hexose-monophosphate (HMP) shunt to generate oxygen-derived free radicals. The effect of tetrandrine on this aspect of neutrophil activity was measured at three points, namely the HMP shunt itself, and the production of superoxide anion and hydrogen peroxide (H_2O_2).

HMP shunt activity was quantitated by the conversion of ^{14}C -1-glucose to $^{14}CO_2$ (Ferrante & Rencis, 1984; Thong & Rencis, 1980). Briefly, 2×10^6 neutrophils were treated with 0.1 $\mu g/ml$ of PMA in a total volume of 1ml at $37^\circ C$ for 45 mins. Then 1N hydrochloric acid was added to drive out any dissolved $^{14}CO_2$ which was captured in 5N sodium hydroxide and quantitated in a scintillation spectrophotometer. Results were expressed as DPM \pm SD of triplicate experiments.

Superoxide anion generation was determined as previously described (Babior et al, 1973; Ferrante et al, 1987). Each 1 ml of 3×10^6 neutrophils was incubated at 37°C for 30 mins. with PMA as the stimulant, and 75 μ M ferricytochrome C in the presence or absence of 10 μ g/ml of superoxide dismutase. The supernatant was removed after centrifugation and absorbance read at 550 nm in a spectrophotometer. Results are expressed as nM superoxide/ 10^6 cells/min. (mean \pm SD) of triplicate experiments.

Hydrogen peroxide production was measured as previously described (Paton & Ferrante, 1983). Briefly, 50 μ l of 5×10^6 /ml of neutrophils was added to each well of a microtitre plate. After incubation at 37°C for 60 min, 20 μ l of 500 μ g/ml horseradish peroxidase (Type II, Sigma Chemical, St. Louis), 70 μ l of phenol and 40 μ l of HBSS were added. The plates were further incubated for 30 mins at 37°C and the reaction stopped by adding 25 μ l of 2N NaOH. The absorbance at 620 nm was measured in a spectrophotometer.

Hypoxanthine-xanthine oxidase reaction

The hypoxanthine-xanthine oxidase system was used to generate oxygen-derived free-radicals. To 6.3 μ M of hypoxanthine was added 0.05 units of xanthine oxidase in the presence or absence of tetrandrine, and the super-

natant assayed for superoxide anion and uric acid as previously described (Ferrante et al, 1984).

Results

Neutrophil locomotion

There was significant inhibition of random movement from 0.72 ± 0.06 mm/2 hr to 0.42 ± 0.03 mm/2 hr at 10 µg/ml concentration of tetrandrine ($p < 0.01$). Similarly, neutrophil chemotaxis was reduced from 2.29 ± 0.06 mm/2 hr to 1.72 ± 0.18 mm/2 hr by 10 µg/ml concentration of tetrandrine ($p < 0.001$). No significant effects occurred at lower concentrations of the drug (Table 11.1)

Phagocytosis

Significant inhibition of phagocytosis occurred at a tetrandrine concentration of 10µg/ml (Table 11.1) with a reduction from $61.1 \pm 4.2\%$ to 30.6 ± 2.6 ($p < 0.001$).

Degranulation

There was a small but significant suppression by tetrandrine of the release of beta-glucuronidase, an enzyme present in specific (secondary) granules, and this occurred when either PMA or FMLP was the stimulant (Table 11.2).

By contrast, there was no inhibition of release of

Table 11.1 Effect of tetrandrine on human neutrophil random movement, chemotaxis and phagocytosis.

Tetrandrine ($\mu\text{g/ml}$)	Adherence-dependent functions (mean \pm S.D.)		
	Random Movement (mm/2hr)	Chemotaxis (mm/2hr)	Phagocytosis (%)
0	0.72 ± 0.06	2.29 ± 0.06	61.1 ± 4.2
0.1	0.80 ± 0.03	2.26 ± 0.08	61.7 ± 4.6
1.0	0.75 ± 0.06	2.25 ± 0.04	51.6 ± 3.1
10	$0.42 \pm 0.03^*$	$1.72 \pm 0.18^{**}$	$30.6 \pm 2.6^{**}$

* $p < 0.01$

** $p < 0.001$

Table 11.2 Effect of tetrandrine on human neutrophil degranulation

Tetrandrine ($\mu\text{g/ml}$)	Neutrophil degranulation (mean \pm S.D.)			
	Lysozyme (% release)		beta-glucuronidase (% release)	
	PMA	FMLP	PMA	FMLP
0	43.9 \pm 2.3	57.8 \pm 2.4	6.5 \pm 0.08	53.1 \pm 0.9
1	44.4 \pm 4.6	62.1 \pm 3.1	5.4 \pm 0.08*	51.4 \pm 1.3
10	38.7 \pm 2.3	53.8 \pm 1.4	4.7 \pm 0.04*	49.4 \pm 1.0**

* $p < 0.001$ ** $p < 0.01$

lysozyme, found in both azurophil (primary) and specific granules (Table 11.2).

Respiratory burst

Although there was dose-dependent inhibition of the HMP shunt and H_2O_2 production by tetrandrine, the values only reached statistical significance at a concentration of $10\mu g/ml$ (Table 11.3)

By contrast, there was marked suppression of superoxide anion production at all 3 concentrations of tetrandrine used in these experiments (Table 11.3). One explanation for this discrepancy would be that tetrandrine has additional properties as a superoxide scavenger. This was investigated in the next set of experiments.

Anti-oxidant effect

As shown in Table 11.4, the generation of superoxide anions was significantly reduced in the presence of $10\mu g/ml$ of tetrandrine. Yet there was no reduction in the amount of uric acid, the end product of the hypoxanthine-xanthine oxidase system (Table 11.4). This indicates that reduction of superoxide anion in the medium was not the result of enzymic inhibition, but rather of superoxide scavenging by tetrandrine.

Table 11.3 Effect of tetrandrine on the respiratory burst of human neutrophils.

Oxygen metabolism (mean \pm S.D.)			
Tetrandrine ($\mu\text{g/ml}$)	HMP Shunt (DPM $^{14}\text{CO}_2$)	Superoxide anion (nM/ 10^6 cells/30 min)	H_2O_2 (μM)
0	16,655 \pm 535	9.40 \pm 0.06	9.8 \pm 1.7
0.1	12,405 \pm 1,428	4.60 \pm 0.07**	N.D.
1.0	12,332 \pm 2,396	3.10 \pm 0.06**	7.5 \pm 0.8
10	9,961 \pm 1,609*	0.78 \pm 0.01**	0.2 \pm 0.2**

N.D. = not done. HMP shunt and superoxide anion production was stimulated by PMA, whilst H_2O_2 production was stimulated by FMLP.

* $p < 0.01$

** $p < 0.001$

Table 11.4 Effect of tetrandrine on the generation of superoxide anion and uric acid by the cell-free hypoxanthine-xanthine oxidase system.

Tetrandrine Conc ($\mu\text{g/ml}$)	Superoxide anion (nMoles/min)	Uric acid (pMoles/min)
0	0.18 ± 0.03	21.2 ± 3.6
1	0.17 ± 0.02	N.D.
10	$0.12 \pm 0.02^*$	21.3 ± 2.5

* $p < 0.05$

Discussion

Neutrophils, the major phagocytic cell of the body, accumulate at sites of inflammation or infection by first adhering to vascular endothelium, and then moving into the tissues in response to chemotactic stimuli. On arrival, they phagocytose particulate matter, release lysosomal enzymes from azurophil and specific granules, and generate oxygen-derived free-radicals. Excessive discharge of lysosomal enzymes and free-radicals are major factors in the tissue destruction of chronic inflammatory diseases. The results of the present study show significant suppression of neutrophil random movement and chemotaxis by tetrandrine. Taken together with our previous findings of inhibition of neutrophil adherence (Seow et al, 1986), it appears that one possible mechanism of action of tetrandrine is interference with recruitment of phagocytes.

This study also showed significant suppression of phagocytosis by tetrandrine. However, degranulation of azurophil (primary) granules was unaffected by tetrandrine. In contrast, tetrandrine caused a small but significant inhibition of release of specific (secondary) granular contents. This differential effect of tetrandrine on degranulation is of some interest as it is known that azurophil and specific granules have

Separate control mechanisms (Wright & Gallin, 1979).

Neutrophils respond to appropriate stimulation with a rapid increase in glucose metabolism via the HMP shunt. A number of reactive oxygen species including superoxide anion, singlet oxygen and hydroxyl radicals are generated, some of which are converted to H_2O_2 (Blake et al, 1987; Green et al, 1979). Our data showed significant suppression of HMP and H_2O_2 production only at 10 ug/ml of tetrandrine, compared with significant inhibition of superoxide production even at 0.1 ug/ml concentration. This marked discrepancy can be explained on the basis of the superoxide scavenging capacity of tetrandrine as shown in experiments with the hypoxanthine-xanthine oxidase system. Thus, not only does tetrandrine interfere with the generation of oxygen-derived free-radicals, it also inactivates them. This anti-oxidant property of tetrandrine may account for a substantial portion of its anti-inflammatory effects.

The anti-phagocytic and anti-oxidant actions of tetrandrine are not due to its toxic effects on neutrophils as shown by dye exclusion studies (Seow et al, 1986) and by measurement of the cytoplasmic enzyme lactic dehydrogenase (data not presented).

It has been estimated that two-thirds of pharmaceuticals in current usage are derived from traditional

or herbal remedies (Sneader, 1986). Contributions from the ethnopharmacy of China has been recently reviewed by Chen (1981). Quinhaosu, a herbal remedy for malaria, is a more recent contribution from this vast storehouse (Klayman, 1985). In this regard, tetrandrine was first isolated in the 1930s (Chen & Chen, 1935; Kondo & Yano, 1932), synthesised in the late 1960s (Inubushi et al, 1969), then evaluated in the 1970s for its modest anti-tumour activity (Creasey, 1976), before finally emerging as a promising anti-inflammatory agent. Data from this and other studies suggest that it has potent anti-phagocytic and anti-oxidant properties, and may warrant further study in animal models of chronic inflammatory, auto-immune, and rheumatic diseases. It may also be useful in ageing, mutagenesis and carcinogenesis, radiobiology and other disease processes where oxygen derived free-radicals may have a role in pathogenesis.

CHAPTER TWELVE

DISCUSSION

Introduction

The work in the thesis was done to explore the possibility that neutrophils perform wider roles in the oral cavity than previously thought. In the three major dental diseases, roles of the neutrophils previously unemphasised were investigated. In dental caries, it was found that the interaction of neutrophils with serotype c strains of *Streptococcus mutans* resulted in suppression of the neutrophils, suggesting that circumvention of these important defence cells may be a significant reason for serotype c to be the most commonly isolated serotype in the oral cavity. With regard to pulp diseases, many pulpotomy medicaments were shown to stimulate neutrophils at low concentrations, indicating their potential for causing pulpal damage through tissue-destroying products released by the neutrophils. With regard to periodontal disease, the direct interactions of common periodontal microbial

pathogens with neutrophils were explored. It was found that *Fusobacterium nucleatum* stimulated neutrophils whereas *Bacteroides gingivalis* and *Actinomyces viscosus* depressed them through mechanisms of direct alteration of surface receptors or the secretion of self-regulatory cytokines.

To further elucidate the roles of the neutrophils in inflammation, their control by lymphokines and monokines were investigated. The studies in this thesis indicated that interferon- γ and tumour necrosis factors α , and β , enhanced neutrophils, whereas interleukins 1 and 2 depressed them. In addition, the effective pharmacological control of neutrophils by a novel anti-inflammatory drug, tetrandrine, was also shown in further investigations.

In the following sections, the results of the investigations in this thesis are brought into perspective in the light of current understanding of the roles of the neutrophils in oral diseases. For clarity, the main oral diseases are discussed in separate sections. In the first section on dental caries, the importance of the neutrophil in defence of the oral

cavity with regard to *Streptococcus mutans* is discussed. In the second section on pulp diseases, the role of the neutrophil in mediating tissue damage in the pulp is emphasised. In the third section on periodontal disease, the importance of the neutrophil in the complex area of immunopathogenesis of periodontal diseases is discussed. Lastly, the control of the neutrophils by a novel anti-inflammatory drug, tetrandrine, is discussed together with possible clinical applications of this potent immunosuppressive agent.

Dental Caries

Introduction

Dental decay or caries is one of the most common infectious diseases of mankind, affecting over 90% of the population. Although there have been recent reports that there is a general decline in dental caries in developed western countries in the past two decades, the reverse is seen in developing countries where there is increasing access to highly refined sugars (Barnes, 1983).

Dental caries is primarily a disease of the hard dental tissues, usually initiating on tooth enamel but may also start on root surfaces on teeth that have gingival recession. Various distinct clinical entities

of dental caries are now recognised. These include nursing bottle caries, a type of rampant decay seen in children on prolonged exposure to sweet fluids from the nursing bottle (Seow, 1987); radiation caries which occurs in xerostomic patients who have been exposed to radiation of the salivary glands; as well as root caries in the elderly (Newbrun, 1984).

The development of dental caries requires (i) cariogenic bacteria capable of rapidly producing acids which decalcify tooth structure, (ii) sugar in the diet which can be metabolised by bacteria to produce acids as well as enhancing their colonisation.

Major host defence factors which may influence the development of dental caries include anti-microbial factors such as neutrophils, and the immunoglobulins IgG and IgA. In addition, salivary factors which neutralise the acids produced by the bacteria are also important.

Association of *Streptococcus mutans* in dental caries

The bacteria responsible for dental caries are part of the complex microbial flora found on the surfaces of the teeth known as dental plaque. *Streptococcus* species play a central role in plaque development (Socransky et al, 1977). Of these, *Strep. mutans* appears to be most important in dental caries. This organism was first

isolated by Clarke in 1924 who recognised its potential for causing decay by its acidogenic and aciduric properties (Clarke, 1924). However, until Orland proved a bacterial aetiology for dental caries, Clarke's observations remained unrecognised (Orland, 1959). Later studies showed that *Strep. mutans* could induce dental caries formation in many animal models (Carlsson, 1967; Hamada et al, 1979; Gibbons & Van Houte, 1975; Michalek et al, 1975).

The association of *Strep. mutans* with human dental caries has been demonstrated in several studies. Loesche et al (1975) found *Strep. mutans* in over 70% of carious fissure lesions while caries-free fissure surfaces exhibited a low incidence of *Strep. mutans*. Later, a longitudinal study showed that the proportion of *Strep. mutans* increased dramatically in plaque prior to and during fissure decay, while the number of *Strep. mutans* was significantly lower in caries-free sites (Loesche & Straffon, 1979). These and other studies (Duchin & Van Houte, 1978; Hamada & Slade, 1980; Lang et al, 1987) indicating the strong association of *Strep. mutans* with human dental caries, as well as the demonstration that this organism is fully virulent in animal models (Carlsson, 1967; Michalek et al, 1975) suggest but do not prove that *Strep. mutans* is of major aetiological significance in human dental caries.

Laboratory studies have shown that the "mutans" group of streptococci is a heterogenous collection of organisms which can be subdivided into several distinct species. The method of classification usually involves serologic reaction of extracted cell wall polysaccharide with antisera prepared against different variants of *Strep. mutans* (Bratthall, 1972). Five serotypes (a - e) were originally isolated (Bratthall, 1972) and two more (f & g) were later added (Perch et al, 1974). Among these serotypes, differences can be detected in cell wall components as well as biochemical and culturing characteristics (Bratthall & Kohler, 1976) including the ability to ferment different types of sugars (Shklair & Keene, 1974). However, no differences in cariogenic potential among the different strains can be firmly determined (Denepitiya & Kleinberg, 1984).

Serotype c is the most prevalent serotype of *Streptococcus mutans*

Extensive epidemiological investigations using mainly immunofluorescence techniques have indicated that serotype c is the most prevalent serotype of *Strep. mutans* in Western industrialised populations (Duany et al, 1972; Bratthall, 1972; Grenier et al, 1973; Bright et al, 1977; Qureshi et al, 1977). In addition, Hamada et al (1976) as well as Masuda et al (1979) also

reported that serotype c is the most commonly isolated serotype of *Strep. mutans* in Japanese children. Other serotypes such as d, e, f and g have been occasionally isolated (Grenier et al, 1973; Berkowitz et al, 1975). It is of interest to note that almost none of serotype a and b strains were found in most of the recent studies, whereas earlier reports indicated their presence in some samples.

Other investigators have also shown, using a biotyping method, that strains similar to serotype c predominated (Keene et al, 1977; Shklair & Keene, 1974; 1976). However, the biotyping method cannot differentiate serotypes c and e (Hamada et al, 1979).

The greater propensity of serotype c to colonise the oral cavity is further shown by the fact that when organisms of serotypes a and c were implanted in humans, serotype a failed to colonise, although a serotype c strain of human origin appeared to do so (Svanberg & Loesche, 1978).

Reasons for the increased prevalence of serotype c

This increased tendency of serotype c over the other serotypes of *Strep. mutans* to colonise the teeth may be due to several possibilities which may act synergistically. Firstly, serotype c may possess

superior biochemical properties that enhance its adhesion, survival and growth in the oral cavity (Svanberg & Loesche, 1978). However, a recent study investigating three main variables of cariogenic potential, ie, acidogenicity, aciduricity and the ability to accumulate and degrade stored polysaccharide (a means of producing a more acidic pH) did not show any particular advantage possessed by serotype c over other serotypes in these aspects (Denepitiya & Kleinberg, 1984).

Instead, it is likely that this serotype of *Strep. mutans* possesses mechanisms to evade host defences. Many streptococcal species are equipped with this capacity, such as anti-phagocytic M protein (Foley & Wood, 1959; Gemmell et al, 1981) and the leukocidins of group A *Streptococci* (Hirsch et al, 1963; Ofek et al, 1970; Sullivan & Mandell, 1980). Our study (Seow et al, 1987c) which shows that serotype c possesses an unique capacity to depress neutrophils, may help explain its increased prevalence over other serotypes in humans. As neutrophils constitute over 90% of the leukocytes in gingival crevicular fluid, and represent the first line of defence against extracellular microbes (Johnson, 1982), their circumvention by serotype c may confer on the organism an added advantage to oral colonisation compared to other serotypes, which have no effect or activate the neutrophils.

Host defence mechanisms in dental caries

Other aspects of local mucosal defence should also be considered in order to view in perspective the role of the neutrophil in defence against *Strep. mutans*. Of importance is the secretory antibody IgA, found in saliva which may prevent oral microorganisms from adhering and colonising the teeth through effecting their agglutination (McGhee & Michalek, 1981). Animal studies have shown that gnotobiotic rats fed with whole cells of *Strep. mutans* selectively induced IgA antibodies secreted in saliva, colostrum and milk with no serum response, and that salivary antibodies to *Strep. mutans* correlated with a reduced smooth surface caries incidence on subsequent challenge to the organism (Taubman & Smith, 1974; Michalek et al, 1976).

However, other workers have suggested that serum IgG transudated through the gingival crevice plays a greater role in protection against dental caries than IgA found in the saliva (Lehner et al, 1975; 1976a; 1976b; 1982). Lehner et al (1976a) reported that monkeys immunised subcutaneously with formalinised *Strep. mutans* in Freund's incomplete adjuvant had protection against dental caries which was correlated with serum IgG titre. In addition, passive transfer of IgG but not IgA fractions of immune serum induced protection against

caries in rhesus monkeys (Lehner et al, 1976a). These investigations suggest that IgG entering through the gingival crevice may facilitate phagocytosis and killing of Strep. mutans by neutrophils (Lehner et al, 1976a). However, a study by Scully & Lehner (1979) showed that only serum from immunised animals are effective in enhancing phagocytosis which increased in parallel with that of the serum IgG antibody titres, as well as associated with a decrease in dental caries. In contrast, serum from control sham-immunised animals showed little or no rise in titre of antibodies to Strep. mutans and supported only minimal phagocytosis (Scully & Lehner, 1978). A later investigation by Scully & Lehner (1979) further demonstrated that sera from monkeys immunised with a serotype c strain of Strep. mutans increased opsonisation, phagocytosis and killing of other strains of Strep. mutans of the same serotype significantly more than other serotypes. Other investigations by McArthur et al, (1976), Baehni et al, (1977) and Passo et al (1980) also showed that the microbicidal activity of human neutrophils against Strep. mutans was increased in the presence of specific immune sera from immunised rabbits.

However, the clinical relevance of these animal investigations and in vitro studies showing the effectiveness of immune serum in enhancing neutrophil activity

against *Strep. mutans* are far from clear. Firstly, all these studies used serum, not gingival fluid, and it remains to be shown that specific opsonic antibody is present in crevicular fluid. Secondly, the quantity of immunoglobulins transuded from the gingival crevice is low, particularly in healthy states (Cimasoni, 1983; Clagett & Page, 1978). Thirdly, the functional integrity of these proteins has recently been questioned (Hsu & Cole, 1985). In addition, antibody directed against *Strep. mutans* is practically nonexistent following natural infection with this bacteria (Hamada & Slade, 1980; McGhee & Michalek, 1981).

Other humoral-mediated mechanisms of host defence such as the complement system also has not been shown to be active against oral bacteria (Sundqvist et al, 1982). In addition, although complement components have been identified in gingival fluids (Brandtzaeg, 1965; Attstrom et al, 1975;), the low levels found (Clagett & Page, 1978; Cimasoni, 1983) suggest that complement-mediated mechanisms of defence against *Strep. mutans* are probably of no great significance.

Thus, direct interaction of neutrophils with *Strep. mutans* assume prominence in the defence of the gingival crevicular area. Yet very few previous studies have examined this interaction. Studies by McArthur and

Taichman (1976) and Taichman & McArthur (1976) indicated that Strep. mutans when grown in culture media devoid of sucrose had little direct effect on neutrophils. However, when grown in sucrose-rich media or coated with dextran, neutrophil enzyme release was triggered. However, as the serotype of Strep. mutans used in these investigations was not mentioned, it is difficult to compare their results with our present data (Seow et al, 1987c).

Colonisation and transmission of Streptococcus mutans

Once a particular strain of Strep. mutans has become established in the mouth, there is great difficulty of colonisation by other strains (Hillman et al, 1985). This indicates that once a serotype c strain is established, it is not usually replaced by other strains. The establishment of Strep. mutans occurs early in life, soon after the eruption of the teeth, and maternal transmission appears to be the chief source of infection (Davey & Rogers, 1984).

In conclusion, our data suggest that the high prevalence of serotype c of Strep. mutans in humans may be due to the fact that this particular serotype possesses an unique ability to suppress neutrophils. As this property of the microorganism resides in its cell wall, further investigations are necessary to isolate

the particular components involved in mediating the response with the neutrophils. Once these are purified, the development of monoclonal antibodies may further elucidate not only the understanding of the interaction of *Strep. mutans* with the neutrophil, but may also have clinical applications in the prevention of this extremely common infection.

Pulpotomy Medicaments

Introduction

The pulpotomy technique involves the removal of the coronal infected and inflamed pulp of a primary tooth, leaving sound, unaffected root pulp intact. The rationale for this technique is to keep a primary tooth in a symptomless state until its normal exfoliation time so that premature extraction is prevented.

Failure of current pulpotomy medicaments to meet histological success

Established medicaments used for dressing the exposed amputated pulp stumps include formocresol, zinc oxide eugenol and calcium hydroxide. Recently, glutaraldehyde has been introduced as a possible less toxic replacement for formocresol (Ranly & Lazzari, 1983; Garcia-Godoy & Ranly, 1987). Collectively, most

clinical studies on pulpotomies using the above medicaments have indicated high success rates based on lack of clinical symptoms and minimal radiographic changes (Law & Lewis, 1964; Doyle et al, 1962; Magnusson, 1978). However, histological studies of teeth often show serious pathological features, notably chronic inflammation, necrosis and internal resorption (Magnusson, 1978; Rolling et al, 1976).

Reasons for poor histological appearance after pulpotomy

The reasons for the poor histological appearance of the teeth after pulpotomy remained poorly explained by previous investigators. Many authors blamed direct toxicity of the medicaments, causing cell death and necrosis of the pulpal tissues (Magnusson, 1980). Others have suggested, instead, that many of the teeth indicated for pulpotomy are already inflamed in the root pulp and that the histological features of chronic inflammation and necrosis may be existing even prior to the pulpotomy (Magnusson, 1970).

One possibility not previously mentioned is that pulpal inflammation and necrosis may be mediated via tissue damaging products of stimulated neutrophils, which are first cells to respond to the irritation. The stimulation may be the result of contamination with bacteria during the pulpotomy procedure but this is

unlikely, as most pulpotomy medicaments have strong bactericidal properties (Magnusson, 1980). More likely, the neutrophil stimulation is the response to the pulpotomy medicament placed directly over the amputated pulp tissue. Seow & Thong, (1986c) have shown that except for glutaraldehyde, the commonly used pulpotomy medicaments, formocresol, eugenol and calcium hydroxide at low concentrations all have the potential of activating the neutrophils. This stimulation probably results in the release of lysozomal enzymes and toxic oxygen radicals which have powerful tissue degrading properties (Weissman, 1972). In addition, various phlogistic substances are released by stimulated neutrophils such as by-products of arachidonic acids which augment and amplify the inflammatory response (Salmon & Higgs, 1987), thus increasing tissue damage.

That the pulpotomy medicaments induce the neutrophil-mediated changes observed in treated pulps is also shown by the fact that our results (Seow & Thong, 1986c) are in clear correlation with recognised histologic changes described in teeth treated with these medicaments (Rolling & Lambjeng-Hansen, 1978; Magnusson, 1970; 1971; Tagger & Tagger, 1984). With formocresol as the medicament, a zone of fixation usually is evident where the pulp is in direct contact with the medicament. Farther away, where the concentration of formocresol is

decreased, there is a zone of poor cellular definition and necrosis. Apical to this is a zone of chronic inflammation which blends into normal tissue (Rolling & Lambjerg-Hansen, 1978). Histologic sections of teeth treated with calcium hydroxide or eugenol also show a zone of tissue necrosis adjacent to these medicaments, followed by a zone of chronic inflammation apically (Magnusson, 1970; 1971). In contrast, glutaraldehyde produces a zone of tissue fixation where it is in direct contact with the pulp, while apical to this is a zone of normal tissue with few inflammatory cells (Tagger & Tagger, 1984; Kopel et al, 1980).

Our study (Seow & Thong, 1986c) showing that glutaraldehyde has least effects on the neutrophils compared with other medicaments, confirms and extends histologic and clinical observations demonstrating its good clinical potential (Kopel et al, 1980; Garcia-Godoy, 1986; Garcia-Godoy & Ranly, 1987). However, while glutaraldehyde may be indicated as the pulpotomy medicament of choice based on these findings, other medicaments, namely calcium hydroxide and eugenol are established medicaments in clinical pedodontics. To improve clinical results using these medicaments, it may be appropriate to consider incorporation of anti-inflammatory agents to preparations of these medicaments to overcome their stimulatory effects on the phagocytic

cells. One commercial preparation containing triamcinolone, a powerful corticosteroid, is already available (Langeland et al, 1977). However, because of the well-known serious side effects of corticosteroids, this preparation is often rejected by many clinicians, particularly for use in children.

The novel anti-inflammatory agent, tetrandrine (Seow et al, 1987a; 1987b) with potential suppressive effects on neutrophils, but little systemic side effects, may be extremely suitable for incorporation into pulpal medicaments as a topical dressing. This possibility is currently being explored.

Periodontal Diseases

Introduction

Chronic periodontal diseases represent a heterogeneous group of inflammatory conditions involving the tooth supporting tissues, namely the gingiva, alveolar bone and periodontal fibres. The most common forms of periodontal diseases include gingivitis where the inflammation is confined to the superficial soft tissues surrounding the teeth, and periodontitis where there is inflammation and destruction of the alveolar bone. Clinically, in adults, periodontal disease may manifest

as a "stable" type which does not lead to tooth loss or the "progressive" type which shows continual destruction of the periodontium (Seymour et al, 1979a). In addition, in children, a form of periodontitis characterised by severe, rapid destruction of the periodontium is seen (Newman, 1980a). These distinct clinical entities of periodontal diseases show different pathologic, biochemical and immunologic patterns, probably resulting from different microorganisms as well as from different host responses in each case.

Bacteriological considerations in periodontal disease

It is now well established that the aetiologic agent in most forms of periodontal diseases is bacterial dental plaque. However, there is difficulty in determining the exact nature of the microbiota associated with periodontal health and disease as many of the organisms found in disease are also isolated from the healthy periodontium (Slots, 1979; Newman, 1980a).

Although early investigators have suggested that it is the general overgrowth of the bacterial mass which leads to disease, it is now well accepted that specific forms of periodontal disease have specific bacterial aetiologies (Loesche, 1976). In the development of gingivitis, there is an increase in supragingival plaque formation, and Gram-positive filaments and rods such as *Actinomyces* appear significant, especially in the early phase of the

disease (Loe et al, 1965). In the later stages of gingivitis, where clinical inflammation is obvious, *Fusobacterium*, *Vibrios* and other Gram-negative motile forms are found (Svanberg et al, 1982).

Chronic periodontitis is associated with an increase in subgingival plaque which consists of a group of microorganisms attached to the tooth surface as well as another group which is unattached and lies between the attached groups and the gingival tissues. The unattached group is probably of greater significance in the aetiology of periodontal disease as the bacteria are in direct contact with the gingival tissues. In most instances, filamentous organisms such as *Actinomyces* species are present in the attached portion of plaque, whereas in the unattached portions, are various Gram-negative organisms such as *Fusobacterium nucleatum*, *Bacteroides gingivalis*, *Wolinella recta*, *Hemophilus* species, *Selenomonas sputigena*, *Capnocytophaga*, *Camphyl-obacter*, as well as *spirochaetes* (Tanner et al, 1979). In localised juvenile periodontitis (periodontosis), the main organisms implicated are *Capnocytophaga*, *Hemophilus actinomycetemcomitans* and *Eikenella corrodens* (van Palenstein Helderman, 1981).

The periodontal pathogens may mediate tissue damage directly by producing exogenous products which have

tissue-degrading properties, eg. collagenase, produced by *Bacteroides* species (Slots & Genco, 1984). In addition, cytotoxins which hamper the functioning of normal defence mechanisms such as that of the neutrophils may be produced (Zambon et al, 1986; Shurin et al, 1976). Also, some bacterial components such as endotoxin can act directly on bone cells to cause resorption (Hopps et al, 1980). However, the extent of direct tissue damage mediated directly through bacterial effects are probably small compared to the damage resulting from host responses evoked through the interaction with bacteria or their products. Bacterial components such as endotoxin from Gram-negative bacteria and peptidoglycans from Gram-positive organisms are powerful activators of the inflammatory response which may result in severe pathologic changes of the periodontium.

Inflammatory responses in periodontal disease

The initial host response following the accumulation of bacteria or their products is migration of the neutrophils into the gingival crevice and a vascular response, resulting in an acute inflammatory exudate and manifesting clinically as acute gingivitis which is observed within about 4 days of the beginning of plaque accumulation (Page & Schroeder, 1976). The inflammatory changes are seen in about 5%-10% of the marginal

gingival connective tissue and in this zone much of the collagen is destroyed, most likely from the activity of collagenase and other enzymes released by infiltrating and transmigrating neutrophils (Schroeder & Attstrom, 1979). If plaque is not removed, the inflammatory response extends deeper into the tissues and a chronic inflammatory infiltrate characterised by lymphocytes and macrophages predominates, along with small numbers of plasma cells located around the periphery of the infiltrate. The acute inflammation persists as evidenced by vasculitis and the presence of neutrophils, especially in the junctional epithelium (Page, 1986). In children, this early lesion progresses to a stable gingivitis characterised by lymphocyte accumulation and minimal tissue destruction (Mackler & Crawford, 1973; Longhurst et al, 1979). In adults, established gingivitis may either remain stable and does not progress for months or years, or alternatively, becomes more active and progresses to destructive lesions (Page et al, 1975). The reasons why some lesions remain stable while others progress to the destructive form are not clear, although microbiological differences as well as differing host responses may contribute to the change. In particular, Seymour et al (1979a) have suggested that stable lesions are associated with predominance of T-lymphocytes while progressive lesions have a predominance of B-lymphocytes.

In the progression of gingivitis to periodontitis, there is alveolar bone destruction, resulting in the formation of periodontal pockets lined with pocket epithelium. In periodontitis characteristic findings are large predominances of lymphocytes and perivascular cords of plasma cells extending towards a hyperplastic pocket epithelium (Okada et al, 1983). The zone of acute inflammation persists along the pocket wall, with neutrophils and monocytes migrating through the epithelium which may remain ulcerated (Seymour & Greenspan, 1979).

Immunopathogenesis of periodontal diseases

1. Possible immune mechanisms in tissue damage

Based on knowledge gained from the immunopathogenesis of other diseases and from histological investigations, four principal mechanisms have been suggested for the participation of the immune system in tissue destruction in periodontal diseases. These are (i) induction by plaque bacteria of a specific T-cell mediated immune response (Wilde et al, 1977); (ii) complement activation by immune complexes formed from antigenic activation of B lymphocytes and production of specific antibody (Kahnberg et al, 1976); (iii) complement activation by the alternative pathway by plaque

bacteria (Allison et al, 1976), and (iv) lymphokine production by activated B and T lymphocytes (Seymour et al, 1979b).

However, in the light of current knowledge, the above mechanisms cannot fully elucidate the pathological changes in the periodontal lesion. Firstly, although the early periodontal lesion manifests characteristics of T-cell mediated hypersensitivity, it is unlikely that this immune mechanism plays a major role, except for a short-lived phase of early gingivitis as well as in the gingiva of deciduous teeth (Page & Schroeder, 1982). In severe disease, B lymphocytes and plasma cells are found rather than T lymphocytes and macrophages, as would be expected if the lesions were a form of delayed hypersensitivity (Seymour & Greenspan, 1979; Lehner et al, 1976b). In addition, Nobreus et al (1974) showed that even after total depletion of circulating T-cells, dogs were still able to develop gingival inflammation, hence the role of T-cells in developing inflammation is questioned.

The immune complex pathway is also unlikely to be of major importance because most of the periodontal pathogens evoke predominantly polyclonal antibodies (Mangan et al, 1983) and the immunoglobulins produced locally have little or no specificity for the antigenic

determinants of the activating bacteria (Page & Schroeder, 1982). Hence, although bacterial products have access to the diseased tissue, immune complex deposits are rarely seen (Genco et al, 1974; Clagett & Page, 1978). In addition, the low levels of complement found in the gingival fluid suggest that complement-mediated mechanisms of tissue damage are probably not of great significance (Schenkein & Genco, 1977).

The production of lymphokines by activated T and B cells is currently thought to play a central role in the immunopathogenesis of periodontal diseases, but understanding of the complex interplay of cells and mediators is still far from complete (Seymour, 1987). Hence, the above current theories on the immunopathogenesis of periodontal diseases fail to explain fully the mechanisms of damage as well as the train of events that occur after the initiation of disease.

2. Neutrophil-bacteria interactions as important determinants of periodontal disease

The interactions of neutrophils with bacteria may hold the key to further understanding of the immunopathogenesis of periodontal diseases. The neutrophil is the first cell to encounter potential periodontal pathogens and the result of this neutrophil-bacteria interaction

may determine the possibility of colonisation of the tooth by the bacteria, the early acute inflammatory changes evoked and even the changes leading to the chronic inflammatory states as well.

(i) Bacterial colonisation

In the early stages of plaque development when few organisms are encountered, it is likely that neutrophils are of central importance in their elimination, as studies have shown that other microbicidal mechanisms such as complement-mediated killing, is ineffective (Sundqvist et al, 1982). Therefore, those microorganisms that can suppress the neutrophils and circumvent this initial and crucial host defence mechanism are able to colonise the teeth with greater ease than those which are unable to do so. Using a few representative periodontopathic bacteria, Seow et al (1987d) [Chap 5, this thesis], showed that *Actinomyces* is one such organism whose suppression of neutrophils may account for its relatively early and successful plaque colonisation (Socransky et al, 1977). The early establishment in plaque of such an organism may then pave the way for the colonisation of other species which have affinity for the glycocalyx of its cell walls (Cisar et al, 1979). Similarly, *Bacteroides* which has been shown to be another organism capable of neutrophil suppression (Genco & Slots, 1984; Seow et al, 1987d;

Chap 5, this thesis), may be thus conferred with an added advantage in tooth colonisation when other growth conditions are favourable. Our studies (Seow et al, 1987d) showed that *Bacteroides* directly inhibits neutrophil adherence as well as induces the secretion of a suppressive self-regulatory cytokine for neutrophils, whereas Van Dyke et al (1982) showed that it can elaborate products which depress neutrophil chemotaxis. Furthermore, it was shown that phagocytosis and killing of an encapsulated *Bacteroides* strain was depressed (Okuda & Takazoe, 1973) and that several strains of *Bacteroides* produce catalase and superoxide dismutase to resist oxygen-dependent intracellular killing mechanisms of the neutrophils (Gregory et al, 1978). In addition, recent evidence that *Bacteroides* species are located in the tissues in periodontal lesions indicates that circumvention of neutrophils may have contributed to their presence (Neiders et al, 1987).

Other periodontopathic pathogens capable of inhibiting neutrophils have also been reported. These organisms are usually associated with severe periodontal destruction, suggesting that loss of this important host defence mechanism may lead to excessive disease. Shurin et al, (1979) showed that peripheral neutrophils from two patients with oral *Capnocytophaga* infections exhibited distinctive abnormalities of neutrophil

morphology and locomotion, which disappeared after eradication of the Capnocytophaga infections. In addition, depression of neutrophil chemotaxis by Capnocytophaga has also been reported in in vitro (Van Dyke et al, 1982) and in vivo (Lindhe & Socransky, 1979).

Another periodontopathic organism well described for its ability to circumvent the neutrophils is *Hemophilus actinomycetemcomitans*. This organism is noted for its ability to produce a leukotoxin which has been suggested as an important virulence factor, but its actual clinical significance is unknown (Zambon et al, 1986). In addition, other workers have isolated factors from culture supernatants of *Hemophilus actinomycetemcomitans* that reduce neutrophil chemotaxis.

Hence, the ability of several periodontopathic bacteria such as *Bacteroides* and *Actinomycetes* to circumvent the neutrophils may be an important advantage aiding their colonisation in the oral cavity.

(ii) Initiation of gingival inflammation and tissue destruction

The release of lysozomal enzymes such as elastase, Cathepsin G, collagenase and gelatinase, as well as toxic oxygen species (Weissman, 1972) released by

neutrophils on interaction with stimulatory periodontal bacteria cause potent destruction of gingival tissues (Narayan & Page, 1983). These mechanisms are probably largely responsible for the initial ulceration of the gingival sulcus in the periodontal lesion (Page & Schroeder, 1976). Animal experiments showed that neutrophil extracts induced a brisk inflammatory response if applied to the gingival margins of dogs (Kahnberg & Hellden, 1977) and that depletion of neutrophils results in substantial decrease in gingival inflammation induced in this manner (Rylander et al, 1975; Kahnberg et al, 1976). In addition, a leukocyte homogenate applied to healthy human marginal gingiva will widen intercellular spaces of pocket epithelium similar to bacterial hyaluronidase (Thilander, 1963). This widening may allow passage of bacterial destructive factors and antigenic components into the connective tissue, evoking further inflammatory changes.

It has been shown that free extracellular neutrophil collagenase and elastase are found in the crevices of inflamed human gingiva (Ohlsson et al, 1974). In contrast, in healthy sites, only a fraction of the enzymes were found, and these were mainly bound to serum protease inhibitors. In other investigations, Kowaski et al (1979) found a significant increase in free mammalian collagenase and neutral protease concentra-

tions in gingival crevices after three weeks of no tooth brushing. As collagen loss is an important feature of periodontal disease, these investigations suggest the involvement of neutrophil enzymes.

The resultant loss of epithelial integrity from damage to gingival crevicular cells due to the neutrophil lysozomal enzymes probably results in ingress of bacteria and their products, causing further stimulation of inflammation and further destruction of tissue. In support of this concept are studies showing that rapid destruction of alveolar bone was the consequence of intensive acute inflammation resulting in ulceration of the junctional epithelium (Schroeder and Lindher, 1980; Kennedy & Polson, 1973). In these studies the infiltrated connective tissue was characterised by rapid bone destruction by osteoclasts and the presence of large numbers of neutrophils. In contrast, in situations where there was no ulceration of the junctional epithelium, a chronic inflammatory infiltrate was observed with few osteoclasts and little alveolar bone destruction. These studies, in addition to the recognised occurrence of bone loss associated with purulent abscess formation, emphasise the importance of neutrophil activity to destruction of alveolar bone (Miller et al, 1984).

While early investigators have reported that whole plaque sonicates are stimulatory to neutrophils (Freedman et al, 1976; Taichman et al, 1966), identification of individual plaque bacteria stimulatory to neutrophils would help elucidate the bacteria involved in the initiating events in periodontal disease. *Fusobacterium nucleatum*, a Gram-negative organism commonly implicated in periodontal disease has been shown to strongly stimulate neutrophil adherence directly as well as induce the secretion of a stimulatory cytokine to enhance the recruitment of other neutrophils (Seow & Thong, 1986b; Seow et al, 1987d, Chap 5 & 6, this thesis).

These investigations confirm and extend the work of Tsai et al (1978) and Taichman et al (1984) showing that *F. nucleatum* induced the release of lysozomal enzymes by neutrophils, as well as that of Passo et al (1982) showing that *F. nucleatum* but not *B. gingivalis* enhanced neutrophil chemiluminescence. These data indicating that neutrophils are strongly stimulated by *F. nucleatum* may explain why severe gingival inflammation is correlated with appearance of this organism in dental plaque (Slots, 1979).

Of great interest is the fact that when *F. nuclea-*

tum is present in coaggregates with other bacteria, its stimulatory potential overrides any depressant effect of other coaggregating bacteria so that plaque with *Fusobacterium* present is always stimulatory to neutrophils (Seow & Thong, 1986b; Seow et al, 1987d; Chap. 5 & 6, this thesis).

(iii) Sustenance of inflammation and immunoregulation

In the early stages of plaque formation when few bacteria are encountered, stimulation of neutrophils probably results in phagocytosis and elimination of the pathogens. However, when plaque formation is established and the organisms become firmly attached to the plaque mass, the neutrophils are probably ineffective in their removal (Freedman et al, 1968; Baehni et al, 1977; Newman, 1980b)

In these circumstances, continual stimulation of the neutrophils will probably result in degranulation of lysozomal enzymes by a process of "frustrated phagocytosis", a well-described phenomenon observed in stimulated phagocytic cells which are unable to ingest irritating particles (Henson, 1971; Wright, 1985). That continued stimulation of neutrophils by plaque bacteria occurs in the periodontium is supported by histological and electron microscopic investigations of plaque-

neutrophil interactions of established plaque, which showed that neutrophils usually form a layer, several cells thick, at close association with the plaque mass (Freedman et al, 1968; Garant, 1976; Baehni et al, 1977; Tsai et al, 1978). These investigations showed that many neutrophils had pseudopodal extensions in the plaque mass and there appeared to be a decrease in lysozomal granules in the neutrophils directly contacting the plaque mass. However, phagocytosis was not a remarkable feature except when individual organisms were encountered (Freedman et al, 1968; Baehni et al, 1977; Tsai et al, 1978).

Therefore, once plaque is established the inability of neutrophils to remove the bacteria completely results in their continued stimulation with release of tissue damaging lysozymes and toxic oxygen radicals. More importantly, stimulated neutrophils produce a cytokine stimulatory for other neutrophils which probably enhances the recruitment of other neutrophils into the inflammatory focus, thus amplifying the response (Seow & Thong, 1986b; Chap 6, this thesis). In addition, they release a neutrophil-immobilising factor (NIF) which inhibits neutrophil random migration and chemotaxis in-vitro (Goetzl & Austen, 1972; Goetzl & Rocklin, 1978; Watt et al, 1983). This factor probably aids in the trapping of neutrophils at the inflammatory site and

prevents their migration.

Stimulated neutrophils are also an important source of arachidonic acid by-products such as prostaglandins which amplify the inflammatory response (Zurier, 1976).

Of further significance is the fact that these various neutrophil products and cytokines released from the bacterial interaction can influence other inflammatory cells so that it is likely that through these mediators, neutrophils exert early control of the inflammatory processes. As the neutrophils are the first cells to be stimulated, their immune mediators released may well determine the pathways and progress of the later inflammatory changes. In this regard neutrophil lysozomal enzymes have been shown to have strong B-lymphocyte mitogenic properties (Vischer et al, 1976; Goto et al, 1984) and stimulate immunoglobulin synthesis (Yamasaki & Ziff, 1976; Panush, 1983). In addition, neutrophil secretory factors have been identified which enhance T lymphocyte responses to mitogens (Nakamura et al, 1976; Yoshinaga et al, 1980; Harris, 1982; Fitzgerald et al, 1983).

Another cytokine now shown to be released by neutrophils (Tiku et al, 1986) which is of great importance in mediating immune functions is interleukin-

1 (IL-1). This molecular mediator induces interleukin-2 (IL-2) receptor expression and thus augments T-cell proliferation (Oppenheim et al, 1986). The presence of IL-1 in gingival fluids (Mergenaghan, 1984) is indicative of its contribution in the inflammatory processes in periodontal diseases.

Other possible immunomodulatory factors released by neutrophils on bacterial stimulation include α -interferon which has wide ranging immunomodulatory activities (Trinchieri & Perussia, 1985). In addition, it is likely that the neutrophil self-regulatory cytokines described by Seow & Thong (1986b) have potential modulatory effects on other immune cells but investigations of these possibilities await the purification of these cytokines.

Thus, considering that the neutrophils are equipped with systems which exert strong influences on other immune cells, it is highly probable that they exert immunomodulatory effects on various inflammatory pathways. With regard to periodontal disease, immunomodulation by neutrophils may determine the transition of acute inflammation to the chronic states. In addition, they may influence the nature of the cellular infiltrate of chronic periodontal lesions. In this regard, whether such lesions become progressive lesions

dominated by B cells or stable lesions dominated by T cells (Seymour et al, 1979a), may well be influenced by immunomodulators released from neutrophils. Indeed, it has already been documented that the conversion of a stable periodontal lesion to a progressive one is accompanied by episodes of acute inflammation (Page, 1986) suggesting that neutrophils may play a role in this conversion process.

The production of lymphokines by activated B and T cells is currently considered an important mechanism of tissue damage in periodontal diseases (Seymour et al, 1979b). These lymphokines are numerous and many are already well characterised. However, with the exception of lymphotoxin (tumour necrosis factor- β), most of these lymphokines do not mediate tissue damage directly. Rather, they influence the activity of effector cells such as the neutrophils and the macrophages (or their derivatives, such as the osteoclasts), which are equipped with potent tissue damaging mechanisms. However, although the effects of some lymphokines, such as the leukocyte inhibitory factor, on neutrophils are well described, (Goetzl & Rocklin, 1978) the effects of others are still unclear. In a series of investigations, we have shown that interferon- γ and lymphotoxin (tumour necrosis factor- β) enhanced the activities of neutrophils whilst IL-2 depressed them (Seow & Thong, 1986b; Seow et al, 1987f, Chaps 7,8, this thesis). In the

1986b; 1987f, Chaps 7 & 8, this thesis). In the periodontal lesions, it is likely that the relative mix and concentrations of these and other cytokines may partially determine the immunobiological transition from acute to chronic inflammation or its resolution. Thus in the early stages of microbial invasion when specific immunological mechanisms are not yet fully mobilised, interferon- γ and TNF- β may promote the recruitment of neutrophils into sites of acute inflammation. Later the secretion of IL-2 would promote the clonal expansion of lymphocytes and retard the ingress of neutrophils, resulting in the lymphocyte predominance characteristic of chronic inflammatory lesions.

Similarly, neutrophils are also influenced by cytokines derived from the other major phagocytic cell of the body, the macrophage. It is likely that these monokines may have either enhancing or suppressive actions on the neutrophils to mediate effective control of these cells. IL-1 secreted by activated macrophages depressed neutrophil adherence, thus encouraging their dissipation (Seow et al, 1987g; Chap 9, this thesis). In contrast, tumour necrosis factor- α activates the neutrophils and encourages their recruitment into the inflammatory focus (Seow et al, 1987g; Chap 9, this thesis). Other monokines secreted probably also have modulatory effects on neutrophil functions, but these

effects have not been systematically studied. In the periodontal lesion it is likely that monokines modulate the neutrophils; however, because of the complex interrelationships of the inflammatory cells, the net effect of these are subject to other cytokines as well.

Prevention and Treatment of Periodontal Diseases

1. Failure of present methods of prevention

Current methods of prevention of periodontal diseases centre mainly on mechanical plaque removal by individuals using the toothbrush and dental floss. This method of disease control has failed for several reasons. Firstly, as plaque begins to form again almost immediately after tooth cleaning, oral hygiene methods need to be practised constantly, thus placing great physical and psychological demands on the individual. Secondly, even when this is attempted, most individuals fail to remove plaque adequately to prevent disease. Thirdly, even though frequent professional tooth cleaning can help control the disease (Axelsson & Lindhe, 1978), costs involved are likely to be excessive and the motivation required of the patients to attend constant professional visits would probably limit attendance to only a few.

Chemical plaque control, an alternative to mechani-

cal plaque control, using various antibacterial substances, eg, chlorhexidine (Addy & Moran, 1983) and antibiotics (Gibson, 1982) also has disadvantages. Firstly, if given systemically, severe side-effects are likely to be encountered. Secondly, if given topically, they need to be delivered subgingivally to be most effective, making it impractical for home use by patients. Therefore, considering the above limitations of mechanical and chemical plaque control, other methods of periodontal disease control are urgently required.

2. Pharmacological method of prevention and treatment

When it was realised that host responses mediate much of the damage in chronic inflammatory periodontal diseases, many investigations were performed to explore the possibility that pharmacological control of host responses may provide the answer to disease control (Rieger, 1987; Vogel, 1985). This was particularly the case as plaque removal methods alone have not been found to be effective.

The potential use of corticosteroids in the control of periodontal inflammation was based on observations that patients taking corticosteroids have less periodontal inflammation compared to age-matched controls (Schuller et al, 1973; Been & Engel, 1982). However,

because of the serious side effects associated with the use of systemic steroids, only the topical application can be considered a suitable method of use in periodontal disease. Several early investigators have reported significant decrease of gingivitis and periodontal inflammation in short-term experiments using topical corticosteroid therapy (Stawinski, 1960; Columbo, 1961; Haim, 1961). More recently, Vogel et al (1984) reported that over a three week study period of experimental gingivitis, a group of patients treated with topical steroids demonstrated less inflammation clinically and histologically compared to a placebo group, although the amount of plaque accumulation was comparable.

The non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting the cyclo-oxygenase pathway which generates the prostaglandins that mediate many destructive inflammatory processes (Lands, 1985). As they have less side effects compared to the corticosteroids, they are considered more suitable for use in the control of periodontal inflammation. However, the choice of an efficacious NSAID appropriate for the periodontium is important as the biochemical characteristics of prostaglandin synthetases derived from various organs may vary considerably and the activity of prostaglandin synthetase inhibitors can be tissue-selective (Flower &

Vane, 1974; ElAttar et al, 1984). Retrospective studies on patients taking NSAIDs such as indomethacin and aspirin for arthritis and spondylitis for at least over one year, showed that they have less gingival inflammation and shallower pocket depths than a control group of matched subjects (Waite et al, 1981; Feldman et al, 1983). However, a prospective study of experimental gingivitis in humans using the NSAID sulindac showed no suppression of gingival inflammation (Vogel et al, 1984). On the other hand, prospective studies in animals indicated that although there was little suppression of gingival inflammation by both topical and systemic indomethacin, there was significant decreases in the amount of bone loss in treated animals (Nyman et al, 1979; Weeks-Dybvig et al, 1982; Vogel et al, 1986).

Thus both the steroids and NSAIDs have been shown to reduce the inflammation seen in periodontal diseases. However, due to the potential serious side-effects accompanying the use of steroids, these do not appear suitable for the control of periodontal inflammation, an incapacitating but non-life-threatening disease. Although the NSAIDs are potentially useful, more experimental work is required before a selective efficacious and safe agent can be recommended for the control of periodontal diseases.

The safety and efficacy of the novel anti-inflammatory drug, tetrandrine appears attractive as an alternative drug for the prevention and treatment of periodontal diseases. The suppression of neutrophils by tetrandrine (Seow et al 1986; 1987b; Chaps 10 & 11, this thesis) would prevent their release of tissue-damaging products which are responsible for much of the initial ulceration of the gingival sulcus in the early lesion. Thus epithelial integrity is preserved, and ingress of bacteria and their products prevented. In addition, suppression of lymphocytes by tetrandrine (Seow et al, 1987a) would prevent release of lymphokines that further activate the effector cells mediating much of the damage in the chronic periodontal lesion.

The pharmacological control of periodontal diseases by tetrandrine is probably augmented by mechanical removal of plaque as well as the simultaneous use of antibacterial substances, as removal of bacteria by these methods will decrease stimulation of the inflammatory processes. However, the most efficacious method of delivery of tetrandrine to the periodontium awaits results of further investigations in animals as well as human clinical trials.

Tetrandrine - a novel anti-inflammatory agent

Tetrandrine is a plant alkaloid isolated from a

traditional Chinese remedy for rheumatic diseases. It is unique in its ability to retard, halt and reverse the fibrosis occurring in silicosis (Li et al, 1981; Liu et al, 1983). In this disease, most of tissue damage is mediated by the lysozomal enzymes and oxygen-derived free-radicals released from macrophages and neutrophils which are stimulated by the non-degradable silica particles lodged in the pulmonary alveoli (Bateman et al, 1982; Bowden & Adamson, 1984). Our investigations have shown that tetrandrine, having strong suppressive effects on neutrophils as well as monocytes, may be effective in controlling silicosis through this mechanism. Tetrandrine significantly inhibited neutrophil adherence, chemotaxis, phagocytosis as well as the secretion of lysozomal enzymes, the activity of the hexose-monophosphate shunt and the production of hydrogen peroxide (Seow et al, 1986; 1987b, Chaps 10 & 11, this thesis). Of great interest is the fact that tetrandrine also shows anti-oxidant properties (Seow et al, 1987b), ie, it can scavenge toxic oxygen radicals, thus increasing its potency as an anti-inflammatory agent.

In other investigations not included in the thesis, we have shown that tetrandrine has other strong in vitro immunosuppressive properties (Seow et al, 1987a). It markedly reduced mitogen-induced lymphoproliferative

responses even when added after the initiation of cultures. Antibody synthesis by B cells was also suppressed as was the natural-killer cell-mediated lysis of the K562 cell-line. Although it does not appear to interfere with receptor-ligand binding, it affects the inositol triphosphate second messenger system, indicating that this may be one of its mechanisms of action at the molecular level (Seow et al, 1987a).

Although further confirmation is required, the low systemic toxicity and the lack of side-effects of tetrandrine are extremely important notable features of this drug. One study of 33 patients, in an open clinical trial for the treatment of silicosis, showed no toxic or undesirable side effects of the drug (Li et al, 1981). Animal studies also report lack of toxic side effects associated with the use of tetrandrine (Yu et al, 1983).

The relative lack of toxicity of tetrandrine appears outstanding when compared with other currently available anti-inflammatory agents such as corticosteroids, penicillamine, gold salts, antimalarials and cyclosporin. The toxic side effects of corticosteroids are well known and include increased susceptibility to infection, adrenal suppression, hypertension and diabetes (Lands, 1985). The use of the penicillamine is

associated with optic neuritis and the nephrotic syndrome (Scheinberg & Sternlieb, 1975), while hepatitis, agranulocytosis, as well as aplastic anaemia are common toxic reactions to gold salts (Rogers & Anderson, 1980). The use of antimalarials is complicated by the serious side effects of retinopathy and blood dyscrasias (Zvaifler, 1979), while treatment with cyclosporin has the risk of lymphoma development and renal damage (Byron, 1987).

The safety and efficacy of tetrandrine thus place it in a strong position to replace many of the current toxic immunosuppressive and anti-inflammatory agents. It is probably most useful in the treatment of neutrophil and macrophage-mediated diseases such as rheumatoid arthritis and the pneumoconioses. In addition, its strong immunosuppressive properties may have wide-ranging therapeutic applications such as in autoimmune diseases and organ transplantation.

The chemical structure and mode of action of tetrandrine suggests that it does not belong to any known class of currently available anti-inflammatory agents. Further biochemical analysis of its activities may even pave the way for the development of a new class of less toxic anti-inflammatory agent that is urgently required today.

Future Directions

The investigations in this thesis have opened up several new avenues of research into the immunopathogenesis of oral diseases as well as the development of a novel anti-inflammatory agent.

As we have shown that the effects of oral bacteria on the neutrophils are mediated through their cell walls, further elucidation of these receptors is pertinent. Identification and purification of these will allow the development of appropriate monoclonal antibodies which will be useful in further investigations elucidating the complex events involved in neutrophil-bacterial interaction. In addition, clinical applications of these monoclonal antibodies to prevent colonisation of organisms responsible for dental caries and periodontal disease may even be possible.

Currently, panels of monoclonal antibodies already prepared against cell wall antigens of *Fusobacterium nucleatum* and *Bacteroides gingivalis* (Bird & Seymour, 1987) are being tested to determine if any of these block the receptors which modulate the effects on neutrophils. The results of these investigations will

help identify bacterial cell components involved in direct bacteria-neutrophil interactions.

In addition, work is now in progress to purify and characterise the self-regulatory cytokines secreted by the neutrophils. These cytokines described by us (Seow & Thong, 1986b) cannot be identified with any known neutrophil cytokines previously described. In particular, the neutrophil-stimulatory cytokine with a molecular weight of 10,000-30,000 daltons can be distinguished from interleukin-1 (IL-1, MW 15,000) by the fact that IL-1 depresses the neutrophil adherence (Seow et al, 1987g), whereas the stimulatory cytokine enhances it. In addition, this cytokine can be distinguished from the phagocytosis-stimulatory factor (PSF, MW, 16,000) isolated by Ishibashi & Yamashita (1981) by the fact that the latter is heat stable whereas our stimulatory cytokine is heat labile (Seow & Thong, 1986b).

The characterisation and purification of the neutrophil cytokines will allow the development of monoclonal antibodies raised against them; these will be useful for further investigations detailing their activity. As well, the production of large quantities of these cytokines by recombinant technology may enable their clinical application in treatment of many medical

conditions where neutrophils play important roles. For example, stimulatory cytokines may be useful in neutropenias and immunocompromised patients in whom stimulation of neutrophils may ensure adequate defence against various pathogens. On the other hand, suppressive cytokines may have useful applications in many chronic inflammatory diseases such as chronic rheumatoid arthritis in which neutrophils play a key role in mediating the tissue damage.

Further investigations are also currently being performed on the novel anti-inflammatory drug, tetrandrine, with regard to its immunosuppressive actions on other inflammatory cells. Preliminary studies show strong immunosuppression on lymphocytes (Seow et al, 1987a) and on histamine release by mast cells (Teh et al, 1987). Studies are also currently being performed to elucidate the mechanisms of action of tetrandrine at a molecular level. These investigations include receptor-ligand binding, protein kinase activity as well as the inositol triphosphate system. In addition, the creation of analogues of tetrandrine are also planned to explore the possibility that some of these may have even greater efficacy and safety than the parent compound.

The potential of tetrandrine for use in chronic inflammatory periodontal diseases, and as a pulp

medicament has already been mentioned. Future studies on animals followed by human clinical trials should elucidate its suitability for clinical dental use.

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